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(54) Title: DNA ENCODING 1,3-BETA-D GLUCAN SYNTHASE SUBUNITS

(57) Abstract

DNA molecules encoding proteins involved in biosynthesis of 1,3-beta-D glucan are identified, cloned, expressed and used in in vitro assays to screen for antifungal compounds, including compounds that affect cell wall biosynthesis. The invention includes but is not limited to the purified DNA molecules, assays employing the DNA molecules, proteins encoded by the DNA molecules, cells expressing the DNA molecules and altered forms of the molecules.

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TITLE OF THE INVENTION

DNA ENCODING 1,3 BETA-D GLUCAN SYNTHASE SUBUNITS

CROSS-RELATED TO OTHER APPLICATIONS

This is a continuation-in-part of U.S.S.N. 08/135,149 filed October 12, 1993, now pending, and a continuation-in-part of U.S.S.N. 08/135,148 filed October 12, 1993, now pending and a continuation-in-part of U.S.S.N. 08/135,150 filed October 12, 1993, now pending, each of which is expressly incorporated by reference.

SUMMARY OF THE INVENTION

DNA molecules encoding proteins involved in biosynthesis of 1,3-beta-D glucan are identified, cloned, expressed and used in in vitro assays to screen for antifungal compounds, including compounds that affect cell wall biosynthesis. The invention includes the purified DNA molecules, assays employing the DNA molecules, proteins encoded by the DNA molecules, cells expressing the DNA molecules and altered forms of the molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a restriction map of plasmid pFF119.

Figure 2 is a restriction map of plasmid pFF334.

Figure 3 is a restriction map of 11.0kb EcoRI insert of pGS3.

Figure 4 is a restriction map of 11.0kb XbaI insert of pGS6. The bold line designates the part of the fksA gene that was sequenced. The insert of pGS15 is shown and its derivatives containing the nested deletions (pGS17-pGS21).

Figure 5 is the DNA sequence and putative amino acid translation of part of the fksA gene.

Figure 6 is the FKS1 DNA sequence.

Figure 7 is the amino acid sequence of FKS1 protein.

Figure 8 is the FKS2 DNA sequence.

Figure 9 is the amino acid sequence of FKS2 protein.

Figure 10 shows the DNA and amino acid sequences of fksA.

Figure 11 shows the DNA sequence of an FKS1 homolog isolated from Candida albicans.

Figure 12 shows the amino acid sequence of an FKS1 homolog of C. albicans.

Figure 13 is a partial list of yeast strains.

BACKGROUND OF THE INVENTION

DNA molecules encoding proteins involved in biosynthesis of 1,3-beta-D glucan are identified, cloned, expressed and used in in vitro assays to screen for antifungal compounds, including compounds that affect cell wall biosynthesis. The invention includes but is not limited to the purified DNA molecules, assays employing the DNA molecules, proteins encoded by the DNA molecules, cells expressing the DNA molecules and altered forms of the molecule.

The present application is directed to purified DNA fragments that contain a gene which reverses the mutant phenotypes of several different strains of Saccharomyces cerevisiae. The gene is called FKS1, for FK506 sensitivity gene 1, and is also known as ETG1 (echinocandin target gene 1). Echinocandins are acyl-substituted cyclic hexapeptides that inhibit the synthesis of 1,3-beta-D-glucan in many fungi. FKS2 is a homolog of FKS1. FKS1 was cloned from a genomic library of Saccharomyces cerevisiae. The properties of FKS1 suggest that it encodes a subunit of 1,3-β-D glucan synthase. Proteins encoded by FKS1 or homologs thereof represent possible targets for drug therapy for fungal disease. The invention includes homologs such as FKS2, which also encodes a target of the echinocandins, and closely-related genes from pathogenic fungi such as Aspergillus fumigatus, Candida albicans and Cryptococcus neoformans.

The invention comprises a gene which reverses the drug-related phenotypes of distinct mutants of S. cerevisiae. Several mutant strains were identified by their altered sensitivity to specific classes of fungal cell wall inhibitors, while another mutant strain is

hypersensitive to the immunosuppressive compounds FK506 and cyclosporin A.

Understanding the mode of action of novel therapeutic compounds employs a variety of experimental approaches involving both biochemistry and genetics. One approach is to try to isolate organisms resistant or sensitive to test compounds. Such mutants can then sometimes be used to isolate genes encoding the drug targets. A general description of some of the relevant areas of yeast biology and the mutant organisms follows.

FK506 and cyclosporin A (CsA) are potent immunosuppressants that inhibit an intermediate Ca^{2+}-dependent step in T cell activation and block interleukin-2 (IL-2) production (for a review, see Sigal et al., 1992, Ann. Rev. Immunol., 10:519-560). FK506 binds to a family of proteins known as FK506 binding proteins (FKBP) while CsA binds to members of another family of proteins called cyclophilins. The resulting drug-receptor complex (FKBP-FK506 or cyclophilin-CsA) binds and inhibits calcineurin, a Ca^{2+}- and calmodulin-dependent protein phosphatase, suggesting that inhibition of calcineurin may be a mechanism of immunosuppression (Liu et al., 1991. Cell, 66:807-815).

FK506 and CsA are also antibiotics that inhibit the growth of certain strains of yeast and fungi. The antifungal properties of these drugs and the existence of FKBP, cyclophilins and calcineurins in yeast and fungi have prompted genetic examinations of the mode of action of the drugs in these organisms.

Using FK506 as a screening agent, hypersensitive mutants were isolated. The fks1-1 mutation discovered in this screen was used to clone the FKS1 gene. A homolog of FKS1 (FKS2) was also discovered and cloned. Examples describing the discovery of this mutation, its use, and the cloning of FKS1, FKS2 and homologs of these genes are provided below.

CsA supersensitive mutants have been reported, but their relationship to FKS1 or FKS2, if any, was not disclosed (Koser, P.K. et al., 1991. Gene, 108:73-80).

The fungal cell wall is a complex structure involved in a variety of vital cellular processes. Vegetative growth, morphogenesis, uptake and secretion of macromolecules and protection against osmotic changes are affected by changes in the composition and integrity of the cell wall. It might be expected that antifungal compounds which act via the inhibition of cell wall synthesis, a process essential to fungi and absent from mammalian cells, would produce an ideal combination of fungicidal activity and low mammalian toxicity.

Efforts from a large number of laboratories have been directed towards the identification of such agents, although compounds of this type have not yet been introduced into clinical practice. The walls of fungi are composed of a number of polymers: chitin, alpha- and beta-glucans, and mannoproteins are all potential targets for antifungal therapy.

A major class of beta-glucan inhibitors is comprised of several lipopeptide antibiotics including aculeacin A, echinocandin B and the pneumocandins. These compounds are all cyclic hexapeptides containing a non-polar fatty acid side chain. Fungicidal activity of the natural products is largely limited to yeasts. Echinocandins are fungicidal by virtue of their ability to inhibit whole cell synthesis of 1,3-beta-D glucan, which disrupts the integrity of the cell wall and causes whole yeast cells to lyse. Echinocandins inhibit in vitro polymerization of glucose into 1,3-beta-D glucan, a reaction that can be catalyzed by mixed membrane fractions from several types of fungi, such as C. albicans, Aspergillus fumigatus and Neurospora crassa.

A second structural class of beta-glucan synthesis inhibitors, the papulacandins and chaetiacandin, contain a glycoside component connected to an aromatic ring system and two long chain fatty acids. These compounds have the same mode of action as the echinocandins. Chemical modification efforts in addition to natural product discovery programs have been aimed at the identification of a clinically useful echinocandin, papulacandin, or chaetiacandin. It is likely that analogues will eventually be incorporated into clinical use.

Matsumoto et al., reported that Pneumocystis carinii, a major cause of pneumonia-related death in AIDS patients in the United States, has beta-glucan in the wall of its cyst form (Matsumoto, Y., et al., 1989. J. Protozool., 36:21S-22S). Inhibitors of beta-glucan synthesis, such as papulacandins and echinocandins, might therefore have efficacy in treating P. carinii infections. Schmatz et al., reported that in a rat model of P. carinii pneumonia, L-671,329 (an echinocandin) and L-687,781 (a papulacandin) were both effective in reducing the number of cysts in the lungs of infected rats (D.M. Schmatz et al., 1990. PNAS, 87:5950-5954). These results suggest that beta-glucan synthesis is a viable target for therapeutics useful in the treatment of P. carinii infections.

There have been several efforts to isolate bona fide drug resistant strains of S. cerevisiae affected in beta-glucan synthesis. Mutants that have been isolated include acu1 (Mason, M., et al., 1989. Yeast Cell Biology meeting, August 15 - August 20, 1989, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Abstract # 154); acr1/2/3/4 (Font de Mora, J., et al., 1991. Antimicrob. Agents Chemother., 35:12 2596-2601); and pap1 (Duran, A., et al., 1992. In: Profiles in Biotechnology (T.G. Villa and J. Abalde, Eds.) Serivicio de Publicaciones, Universidad de Santiago, Spain. pp. 221-232). One disadvantage of these attempts was the poor potency of aculeacin and papulacandin against S. cerevisiae.

In the present work, a more potent echinocandin (L-733,560) was used as a selective agent, and mutants specifically affected in glucan synthesis were isolated. The first mutant discovered in this screen (strain R560-1C) was used to clone the FKS1 gene. A second mutant identified in the search for L-733,560-resistant strains was found to be echinocandin-resistant and supersensitive to the chitin synthase inhibitor nikkomycin Z. Chitin, like beta-glucan, is a polysaccharide essential for the structural integrity of the fungal cell wall. Nikkomycin Z inhibits cell growth and the in vitro polymerization of chitin. The second mutant was also used to clone the FKS1 gene.

DETAILED DESCRIPTION OF THE INVENTION

DNA molecules encoding proteins involved in biosynthesis of 1,3-beta-D glucan is identified, cloned, expressed and used in in vitro assays to screen for antifungal compounds, including compounds that affect cell wall biosynthesis. The invention includes but is not limited to the purified DNA molecules, assays employing the DNA molecules, proteins encoded by the DNA molecules, cells expressing the DNA molecules and altered forms of the molecule.

The present invention relates to the isolation, characterization, expression, and sequence of a DNA molecule encoding S. cerevisiae FK506 sensitivity gene1 (FKS1), which is also known as ETG1, and homologs of FKS1, which include but are not limited to FKS2. The FKS1 gene is obtained from a strain of S. cerevisiae which is capable of producing FKS1 protein. Such strains of yeast are well-known in the art and include, but are not limited to, S. cerevisiae W303-1A, S288C, GRF88, and YFK007.

The FKS2 gene was found in Southern blots of S. cerevisiae genomic DNA as a band hybridizing to a probe consisting of FKS1 DNA.

Although one cannot predict that a particular mutant which is resistant or hypersensitive to these drugs may be isolated, nevertheless, the techniques of isolation of drug hypersensitive or resistant mutants are similar to those used in the isolation of auxotrophic, temperature-sensitive, and UV-sensitive mutants as described in MYG (infra). The FKS1 gene or homologs of FKS1 may be isolated from a chromosomal DNA library by a variety of methods including: (1) complementation of a mutation (fks1-1) rendering cells hypersensitive to the immunosuppressant drugs FK506, cyclosporin A, or other calcineurin inhibitors; (2) complementation of a mutation (fks1-2) rendering cells resistant to echinocandins; or (3) complementation of a mutation (fks1-4) rendering cells hypersensitive to nikkomycin Z. (GYG, infra, pp. 195-230).

The FKS1 gene or its homologs may be isolated from chromosomal DNA by preparing a library of DNA fragments in a DNA

cloning vector and screening individual clones for the presence of FKS1. For example, a library of S. cerevisiae genomic DNA from strain GRF88 in the plasmid YCp50 can be obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, as ATCC 37415.

A plasmid library may be prepared by isolating chromosomal DNA from pure cultures of the microorganisms. Such microorganisms include, but are not limited to, S. cerevisiae strains W303-1A, S288C, GRF88, and MY2146 (YFK007). The chromosomal DNA is fragmented, for example, by partial digestion with one or more restriction endonuclease enzymes, such as BamHI, ClaI, BclI, BglII, KpnI, Sau3AI, or XhoI, with Sau3AI being preferred. The digested DNA fragments are separated by size, and the size specific fragments, about 2 to 15 kb in length, are inserted into a cloning vector.

Cloning vector as used herein is defined as a DNA sequence which allows the incorporation of specific experimental DNA, with the combined DNA being introduced into a host cell that can exist in a stable manner and express the protein dictated by the experimental DNA. The foreign DNA combined with the vector DNA constitutes a recombinant DNA molecule which is derived from recombinant technology. Cloning vectors include, but are not limited to, plasmids, bacteriophage, viruses, and cosmids.

The cloning vector is cut with a restriction endonuclease such as SalI, treated with phosphatase and the DNA fragments are ligated with a DNA ligase, with T4 DNA ligase being preferred. The cloning vectors are used to transform host cells competent for the uptake of DNA. Host cells for cloning, DNA processing, and expression include but are not limited to bacteria, yeast, fungi, insect cells and mammalian cells, with the preferred host being Escherichia coli. The most preferred hosts are E. coli K-12 strains RR1, HB101, JM109, DH11S, or DH5a. When about 5 x 10^4 independent genomic DNA fragments are ligated into a cloning vector, this is called a library. A true library is likely to contain a representation of the entire genome. Examples of such libraries are described in Rose et al., (GYG, infra).

Competent host cells which take up and stably maintain a recombinant DNA molecule in the transformation procedure can be identified by their ability to grow on LB medium supplemented with a plasmid-selective drug. For plasmid vectors containing the ampicillin resistance gene, ampicillin is the preferred selective drug. To obtain a full representation of the library, transformation mixtures are spread on the surface of many agar plates and incubated under appropriate conditions. Transformant cells can be resuspended from the surface of agar plates in a small volume of liquid medium, with 10 ml of LB medium being preferred. The cell suspension is used to inoculate a larger volume of LB liquid, supplemented with the selective drug, and incubated overnight at 37°C. Plasmid DNA is then extracted from the cells by methods known in the art.

Screens to identify the FKS1 gene or its homologs in the plasmid library can be devised. One strategy requires the use of an echinocandin-resistant mutant of S. cerevisiae, such as strain R560-1C (MY2140). Cells are made competent to take up DNA and are then transformed with library DNA. Transformants bearing the FKS1 gene will exhibit a plasmid-dependent decrease in resistance to a selective echinocandin. This expectation is based on information from a genetic analysis of strain R560-1C. When R560-1C is mated to wild-type strains, the heterozygous diploids are intermediate in echinocandin sensitivity compared to the respective parents, suggesting that a single copy of the wild type gene can make the mutant more sensitive to echinocandins.

Aliquots of the transformation mixture are plated on media which are selective for transformants. After incubation to allow growth, colonies are collected, pooled, and stored, preferably by freezing at -80°C in medium supplemented with 25% glycerol. The titer, defined as the number of colony forming units per milliliter, is determined by methods known in the art.

Identification of transformants that contain the FKS1 gene may be accomplished by plating the library onto agar plates containing plasmid-selective medium such that a countable number of colonies

grow on each plate. A portion of each colony is transferred to two agar plates by replica plating: the first plate contains plasmid-selective medium supplemented with a concentration of the selective echinocandin which kills the cells with intermediate sensitivity, and the second contains plasmid-selective medium only. Positive clones are defined as those colonies which grow normally on the plate without echinocandin but grow poorly or not at all on the echinocandin-containing plate.

The echinocandin-sensitive phenotype may be detected by a variety of tests. In one test, cells from a colony are patched directly onto the surface of plates containing different concentrations of the selective echinocandin; cells that grow poorly are scored after two days of incubation.

In a second test, a portion of each colony is transferred by replica plating to an agar plate containing the selective echinocandin at a concentration approximately twice that used in the first test. Positive clones do not grow on these plates.

In a third test, cells from an individual colony are inoculated into plasmid-selective liquid medium and grown to saturation. An aliquot of the saturated culture is used to inoculate fresh liquid medium supplemented with or without the selective echinocandin. After incubation, growth is measured by optical density at a wavelength of 600 nm. Colonies that fail to grow in the presence of echinocandin are scored as positive for increased sensitivity to echinocandin.

In another test, potential clones are assayed in a broth microdilution assay, wherein a range of concentrations of the selective echinocandin are tested. Positive clones are more sensitive to the selective echinocandin than the original resistant mutant.

Tests such as those described above may be used screen a library of genomic DNA so as to identify a recombinant plasmid that contains a functional copy of the FKS1 gene. To determine whether the increase in sensitivity to echinocandin is due to a plasmid-encoded copy of FKS1, positive clones are cured of plasmid DNA and tested for a decrease in sensitivity to echinocandin. If decreased echinocandin resistance is due to the presence of the plasmid, then plasmid loss results

in the loss of this phenotype. Echinocandin sensitivity may be measured in a variety of ways, preferably by the broth microdilution assay.

More direct proof that the increase in sensitivity to echinocandin is due to the presence of a plasmid containing the FKS1 gene may be obtained by isolating plasmid DNA from a positive clone. Cells of E. coli competent to take up DNA are transformed with the plasmid, and transformants are identified and isolated. Plasmid DNA is isolated from the transformed E. coli and then digested with restriction endonucleases to yield fragments of discrete sizes. The size of each fragment can be estimated by conventional methods, such as gel electrophoresis. By digesting the plasmid with a variety of enzymes, a map indicating positions of cleavage is generated; the map is distinct and specific for the cloned fragment. A detailed restriction map is sufficient to identify a particular gene within the genome. Fragments of the cloned gene, generated by digestion with endonucleases, can be purified from agarose gels and ligated into vectors suitable for sequencing by methods known in the art. Such vectors include, but are not limited to pBR322, YEp13, YEp24, pGEM3Zf(+), pGEM5Zf(+), and pGEM7Zf(+), with pGEM3Zf(-), and pGEM7Zf(-) being preferred. Double stranded DNA is prepared from each of the plasmids and used for sequencing.

A second strategy for identifying clones containing the FKS1 gene utilizes its ability to complement an FK506 hypersensitive mutation. An FK506 hypersensitive mutant is transformed with library DNA. Transformants no longer hypersensitive to FK506 are identified by incubating all transformants in the presence of levels of FK506 inhibitory to the growth of the hypersensitive mutant but not to the wild-type strain. Only strains containing DNA comprising the FKS1 gene grow. A similar strategy may be devised using cyclosporin A or any other calcineurin inhibitor to which the mutant is hypersensitive.

A third strategy for identifying clones containing the FKS1 gene utilizes its ability to complement a mutation conferring hypersensitivity to nikkomycin Z. The nikkomycin Z sensitive mutant, such as MS14, is transformed with library DNA. Transformants no

longer hypersensitive to nikkomycin Z are identified by incubating all transformants in the presence of levels of nikkomycin Z inhibitory to the growth of the hypersensitive mutant but not to the wild-type strain. Only strains containing DNA containing the FKS1 gene grow.

The FKS2 gene, a homolog of FKS1, may be isolated from chromosomal DNA. Chromosomal DNA is isolated from pure cultures of microorganisms known from Southern hybridization analysis to contain FKS2, using standard methods. The chromosomal DNA is fragmented by digestion with various enzymes. The isolation of FKS2 may be carried out with the use of a probe consisting of a DNA molecule with a region of nucleotide sequence similar to a portion of that of the FKS1 gene. The length of this fragment need only be great enough to confer specificity for FKS2 in a hybridization screen of DNA from an FKS2 containing organism. This fragment may also be longer than the minimum length required to achieve specificity of hybridization. Preferred fragments are the 3.5-kb KpnI FKS1 fragment or the 10-kb PstI-SphI FKS1 fragment.

The FKS1 or FKS2 gene of S. cerevisiae may be used to isolate and characterize homologous genes in pathogenic fungi. Southern blot hybridization analyses show that genes closely related to FSK1 and FKS2 exist in the pathogenic fungi. Because the pathogenic fungi, which include but are not limited to strains of C. neoformans, C. albicans, A. fumigatus, Magnaportha grisea, and Ustilago maydis, have 1,3-beta-D glucan in their cell walls, it is likely that a functional homolog of FKS1 or FKS2 exists in each of these fungi. It is also likely that a functional homolog of FKS1 or FKS2 exists in other organisms that have 1,3-beta-D glucans in their cell walls. Examples of such organisms include, but are not limited to Pn. carinii.

FKS1 and FKS2 homologs may be detected by isolating chromosomal DNA from C. albicans, C. neoformans, A. fumigatus, A. nidulans, M. grisea, and U. maydis. A portion of the chromosomal DNA is cut to completion with a number of restriction enzymes, such as EcoRI, HindIII, EcoRV, ClaI, and XhoI. The digested fragments of DNA are separated by gel electrophoresis. The fragments are then

transferred to a solid membrane support such as nitrocellulose or nylon membrane with nylon membrane being the preferred method. The nylon blot is then hybridized with a labeled probe. The probe may be labeled with a radioisotope. The radioisotope of choice is ^{32}P. A DNA fragment can be radiolabeled either by a nick translation procedure (such as the one described in Rigby et al., (1977) J. Mol. Biol., 113:237-251) or a random priming procedure (such as the one described in Feinberg and Vogelstein (1983) Anal. Biochem., 132:6-13), with the random priming procedure being preferred. The blot is hybridized overnight with a radiolabeled fragment, the 1.4-kb SalI-ClaI FKS1 fragment or the 3.5-kb SalI-ClaI FKS1 fragment or the 1.7-kb PstI-BglII FKS2 fragment being the preferred probes. The following day the blot is washed and then exposed to XAR-5 film and developed. The conditions for washing the blot are such that only genes with a high degree of homology will hybridize with the probe and appear on the autoradiogram. The size and pattern of the digested fragments which hybridize with the probe generate a genomic map. For each organism, the map is sufficient to specifically identify the FKS1 or FKS2 homologs in the chromosome.

Mutations of the FKS1 gene, including, but not limited to, fks1-1 or disruptions or deletions of FKS1, are useful for screening for glucan synthase inhibitors. Such a screen relies on the change in susceptibility of such mutations compared to an FKS1 wild-type strain to glucan synthase inhibitors. Any technique capable of detecting this difference can be used. A zone of inhibition assay on agar plates is particularly useful.

It is known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those DNA sequences which contain alternative codons which code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein which do

not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally-occurring peptide. Methods of altering the DNA sequences include, but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand.

As used herein, a "functional derivative" of a modified FKS1 DNA or protein is a compound that possesses a biological activity (either functional or structural) that is substantially similar to the biological activity of the FKS1 DNA or protein. The term "functional derivative" is intended to include the "fragments," "variants," "degenerate variants," "analogs" "homolog" or to "chemical derivatives." The term "fragment" is meant to refer to any polypeptide subset of FKS1 protein. The term "variant" is meant to refer to a molecule substantially similar in structure and function to either the entire protein or to a fragment thereof. A molecule is "substantially similar" to a modified protein if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity, they are considered to be variants even if the structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical.

The term "analog" refers to a molecule substantially similar in function to either the entire protein or to a fragment thereof.

"Substantial homology" or "substantial similarity", when referring to nucleic acids means that the segments or their complementary strands, when optimally aligned and compared, are identical with appropriate nucleotide insertions or deletions, in at least 75% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize to a strand or its complement.

The nucleic acids claimed herein may be present in whole cells or in cell lysates or in a partially purified or substantially purified form. A nucleic acid is considered substantially purified when it is purified away from environmental contaminants. Thus, a nucleic acid sequence isolated from cells is considered to be substantially purified when purified from cellular components by standard methods while a chemically synthesized nucleic acid sequence is considered to be substantially purified when purified from its chemical precursors.

Nucleic acid compositions of this invention may be derived from genomic DNA or cDNA, prepared by synthesis or by a combination of techniques.

The natural or synthetic nucleic acids encoding the 1,3-beta-D-glucan synthase subunit of the present invention may be incorporated into expression vectors. Usually the expression vectors incorporating the 1,3-beta-D-glucan synthase subunit will be suitable for replication in a host. Examples of acceptable hosts include, but are not limited to, prokaryotic and eukaryotic cells.

The phrase "recombinant expression system" as used herein means a substantially homogenous culture of suitable host organisms that stably carry a recombinant expression vector. Examples of suitable hosts include, but are not limited to, bacteria, yeast, fungi, insect cells, plant cells and mammalian cells. Generally, cells of the expression system are the progeny of a single ancestral transformed cell.

The cloned 1,3-beta-D-glucan synthase subunit DNA obtained through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant 1,3-beta-D-glucan synthase subunit using standard methods.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be

used to express eukaryotic genes in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells, fungal cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells or bacteria-fungi or bacteria-invertebrate cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant 1,3-beta-D-glucan synthase subunit in mammalian cells. Commercially available mammalian expression vectors which may be suitable for recombinant 1,3-beta-D-glucan synthase subunit expression, include but are not limited to, pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo (342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and λZD35 (ATCC 37565).

A variety of bacterial expression vectors may be used to express recombinant 1,3-beta-D-glucan synthase subunit in bacterial cells. Commercially available bacterial expression vectors which may be suitable for recombinant 1,3-beta-D-glucan synthase subunit expression include, but are not limited to pET11a (Novagen), lambda gt11 (Invitrogen), pcDNAII (Invitrogen), pKK223-3 (Pharmacia).

A variety of fungal cell expression vectors may be used to express recombinant 1,3-beta-D-glucan synthase subunit in fungal cells. Commercially available fungal cell expression vectors which may be suitable for recombinant modified 1,3-beta-D-glucan synthase subunit

expression include but are not limited to pYES2 (Invitrogen), Pichia expression vector (Invitrogen).

A variety of insect cell expression vectors may be used to express recombinant 1,3-beta-D-glucan synthase subunit in insect cells. Commercially available insect cell expression vectors which may be suitable for recombinant expression of 1,3-beta-D-glucan synthase subunit include but are not limited to pBlue Bac III (Invitrogen), as well as pAcUW1 and pAc5G1 (PharMingen).

An expression vector containing DNA encoding 1,3-beta-D-glucan synthase subunit may be used for expression of modified 1,3-beta-D-glucan synthase subunit in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such as E. coli, fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to Drosophila and silkworm derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, lipofection, protoplast fusion, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce 1,3-beta-D-glucan synthase subunit. Identification of recombinant 1,3-beta-D-glucan synthase subunit expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti-1,3-beta-D-glucan synthase subunit antibodies.

Expression of 1,3-beta-D-glucan synthase subunit DNA may also be performed using in vitro produced synthetic mRNA or native mRNA. Synthetic mRNA or mRNA isolated from 1,3-beta-D-glucan synthase subunit producing cells can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being preferred.

The term "substantial homology", when referring to polypeptides, indicates that the polypeptide or protein in question exhibits at least about 30% homology with the naturally occurring protein in question, usually at least about 65% homology.

The 1,3-beta-D-glucan synthase subunit may be expressed in an appropriate host cell and used to discover compounds that affect 1,3-beta-D-glucan synthase subunit.

The present invention is also directed to methods for screening for compounds which modulate the expression of DNA or RNA encoding 1,3-beta-D-glucan synthase subunit or which modulate the function of 1,3-beta-D-glucan synthase subunit protein. Compounds which modulate these activities may be DNA, RNA, peptides, proteins, or non-proteinaceous organic molecules. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding 1,3-beta-D-glucan synthase subunit or the function of 1,3-beta-D-glucan synthase subunit protein. Compounds that modulate the expression of DNA or RNA encoding 1,3-beta-D-glucan synthase subunit or the function of modified 1,3-beta-D-glucan synthase subunit protein may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample.

Kits containing 1,3-beta-D-glucan synthase subunit DNA, antibodies to 1,3-beta-D-glucan synthase subunit, or 1,3-beta-D-glucan

synthase subunit protein may be prepared. Such kits are used to detect DNA which hybridizes to 1,3-beta-D-glucan synthase subunit DNA or to detect the presence of 1,3-beta-D-glucan synthase subunit protein or peptide fragments in a sample. Such characterization is useful for a variety of purposes including but not limited to forensic, taxonomic or epidemiological studies.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen and measure levels of 1,3-beta-D-glucan synthase subunit DNA, 1,3-beta-D-glucan synthase subunit RNA or 1,3-beta-D-glucan synthase subunit protein. The recombinant proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and typing of 1,3-beta-D-glucan synthase subunit. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant 1,3-beta-D-glucan synthase subunit protein or anti-modified 1,3-beta-D-glucan synthase subunit antibodies suitable for detecting 1,3-beta-D-glucan synthase subunit. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Pharmaceutically useful compositions comprising modulators of 1,3-beta-D-glucan synthase subunit activity, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration.

The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds identified according to this invention as the active ingredient can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well

known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

Biologically pure samples of S. cerevisiae MY2095 (YFK007), S. cerevisiae MY2140 (R560-1C), S. cerevisiae MY2147 (YFK532-7C), S. cerevisiae MY2148 (YFK798), S. cerevisiae MY2256 (YMO148, YFK0978), S. cerevisiae MY2257 (YFK1088-23B), S. cerevisiae MY2258 (YFK1088-16D), S. cerevisiae MY2259 (YFK1087-20B), S. cerevisiae MY2260 (YFK1087-20A), and DNA of plasmids pFF119 and pFF334 have been deposited in the permanent collection of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland.

The following examples are provided to further define the invention without, however, limiting the invention to the particulars of these examples.

EXAMPLE 1

Recipes for media used in this work include, but are not limited to the following.

a. YEPD medium

Bacto Yeast Extract	10 g
Bacto-Peptone	20 g
Dextrose	20 g
Distilled Water to 1 liter	
Sterilize by autoclaving.	

For solid YEPD medium, add Bacto-agar to 2% (20 grams) before autoclaving.

b. YPAD medium

Bacto Yeast Extract	10 g
Bacto-Peptone	20 g
Dextrose	20 g
adenine sulfate	60 - 80 mg
Distilled Water to 1 liter	
Sterilize by autoclaving.	

For solid YPAD medium, add Bacto-agar to 2% (20 grams) before autoclaving.

c. YPAD/10 mM $CaCl_2$

Dilute 1 part sterile 1 M $CaCl_2$ into 100 parts YPAD.

d. YPAG medium

YPAD with glycerol (20 g/liter) in place of dextrose

e. <u>SC medium</u>

Bacto Yeast Nitrogen Base without amino acids	6.7 g
Dextrose	20 g
Complete Amino acid powder	0.87 g

Distilled water to 1 liter
Sterilize by autoclaving.

For solid SC medium, add Bacto-agar to 2% (20 grams) before autoclaving.

f. <u>Complete Amino Acid powder</u>

0.8 g	Adenine
0.8	L-Arginine
4.0	L-Aspartic acid
0.8	L-Histidine
1.2	L-Isoleucine
2.4	L-Leucine
1.2	L-Lysine
0.8	L-Methionine
2.0	L-Phenylalanine
8.0	L-Threonine
0.8	L-Tryptophan
1.2	L-Tyrosine
0.8	Uracil
6.0	L-Valine

Mix with a mortar and pestle.

g. <u>Dropout powders</u> are prepared by omitting one or more components from Complete Amino Acid powder. For example, Trp dropout powder is identical to Complete Amino Acid powder except that L-tryptophan is not added.

h. Solid SC medium containing FK506 is prepared by addition of FK506 to autoclaved SC medium when it had cooled to 50-52°C. The medium is dispensed into petri dishes and allowed to solidify. Solid SC medium containing L-733,560 is prepared in an analogous fashion.

i. Trp dropout/dextrose medium

0.87 g	Trp dropout powder
6.7 g	Yeast Nitrogen Base w/o amino acids
20 g	dextrose
1000 ml	distilled water to volume.

Adjust to pH 5.8 with 5 M KOH.
Trp dropout plates are made with 20g/l agar.
Sterilize by autoclaving.

j. Uracil dropout/sorbitol medium

0.87 g	Uracil dropout powder
6.7 g	Yeast Nitrogen Base w/o amino acids
20 g	dextrose
182 g	sorbitol
1000 ml	distilled water to volume

Adjust to pH 5.8 with 5 M KOH.
Sterilize by autoclaving.

k. Uracil dropout/sorbitol agar
Add 20 g/l agar to uracil dropout/sorbitol medium before autoclaving.

l. Uracil dropout/sorbitol soft agar
Add 6 g/l agar to uracil dropout/sorbitol medium before autoclaving.

m. Trp dropout/glycerol
As trp dropout/dextrose but with 20g/l glycerol replacing dextrose.

n. LB medium and LB medium with ampicillin are prepared essentially according to the methods described in Maniatis (infra).

Strains and DNA were isolated and handled by standard procedures (J. Sambrook, E.F. Fritsch, and T. Maniatis, "Molecular Cloning, A Laboratory Manual", second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y. (1989), referred to as Maniatis; and "Current Protocols in Molecular Biology", F.M. Ausubel et al., editors, John Wiley & Sons, New York (1987), referred to as Current Protocols). Many of the procedures for working with S. cerevisiae are described in M.D. Rose, F. Winston, and P. Hieter, "Methods in Yeast Genetics: a Laboratory Course Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y. (1990), referred to as MYG, and in C. Guthrie and G.R. Fink, editors, Methods in Enzymology, Volume 194, "Guide to Yeast Genetics and Molecular Biology", Academic Press, Inc., New York (1991), referred to as GYG.

LIST OF STRAINS

Strain Name	Relevant Properties	MY No.	ATCC
YFK005	MATalpha FKS1 (wt)	MY2094	74059
YFK007	MATa FKS1 (wt)	MY2095	74060
YFK093	MATa FKS1 fkr3 (506^R)	MY2088	74055
YFK132	MATa fks1-1 (506^S)	none	
YFK531-5A	MATalpha fks1-1 (506^S)	none	
YFK532-7C	MATa fks1-1 (506^S)	MY2147	
YFK532-10B	MATa fks1-1 (506^S)	none	
YFK798	MATa fks1-1/YEp-A2B	MY2148	
YFF2409	MATa fks1-5::HIS3	none	
YFF2411	MATa fks1-6::HIS3	none	
W303-1A	MATa FKS1 (wt)	MY2141	
W303-1B	MATalpha FKS1 (wt)	none	
R560-1C	MATa fks1-2 (560^R)	MY2140	
X2180-1A	MATa FKS1 (wt)	MY2136	
MS10	MATa fks1-3 (560^R)	MY2144	
MS14	MATa fks1-4 (560^R, nik^S)	MY2145	

D1-22C	MATa fks1-4 (560^R, nik^S)	none
GG100-14D	MATalpha FKS1(wt)	none

PLASMIDS

Plasmid	Description	Source of cloned DNA
pFF119	FKS1 clone in YCp50	GRF88
pJAM54	FKS1 clone in YEp24	YFK093
pMS10	FKS1 clone in YCp50	GRF88
pFF250	1.7-kb BglII-PstI FKS2 fragment	YFK007
pFF334	10-kb EcoRI FKS2 fragment	S288C

Additional strains and plasmids are shown in the figures.

EXAMPLE 2

Liquid broth microdilution assay

To quantitate the sensitivity of a particular strain of S. cerevisiae to a compound such as FK506, L-733,560, or nikkomycin Z, the following procedure was followed:

Day 1:

Inoculate the strain(s) into 2 ml of SC medium or SC medium substituting a particular dropout powder if selection for an auxotrophic marker (e.g., ura3, his4, etc.) is required. Grow overnight at 30°C with gentle agitation.

Day 2:

Subculture 20 mcL of each overnight strain into 2 ml of fresh medium; incubate for 4-6 h at 30°C.

Seed a sterile flat bottom 96-well, twelve column microtiter plate with 75 mcL of SD or SD dropout medium in columns 2 through 12. *In column 1, seed 150 mcL of the medium.*

Dissolve drug of interest at 4X the desired initial concentration. For L-733,560, a 64 mcg/ml solution in sterile SD is

prepared. Aliquot 75 mcL of the drug suspension into column 3. Using a multichannel pipettor, transfer 75 mcL from column 3 into column 4, pipet up and down three times to mix, and then transfer 75 mcL from column 4 into column 5. Repeat the serial dilution until column 12 is reached; after mixing, discard 75 mcL to waste.

Label 5 ml sterile tubes with each strain to be tested. Aliquot 2 ml of the appropriate media into each tube. Read the A600 of the strains, and dilute the cultures such that the final OD will be 0.0014. For example, if the A600 of a strain is 0.7041, subculture 4 mcL into 2 ml of the media.

Inoculate each strain in a given row by adding 75 mcL of the inoculum into columns 2 to 12. Do not add cells to column 1, as column 1 is the blank. Column 2 serves as the no-drug or 100% growth control. The plate is then incubated overnight at 30°C without shaking.

Day 3:

After approximately 24 hours of incubation, gently agitate the plate to resuspend the cells and read the absorbance at 600nm wavelength. The % control growth for any given well can be calculated by dividing the absorbance value for that well by the value from column 2 in the same row. If this is done for each column, the data can be plotted as "Percent control growth" vs. "Drug concentration". The resulting dose-response curve can be used to compare the drug sensitivities of various strains.

EXAMPLE 3

Isolation of YFK132, an fks1-1 mutant

S. cerevisiae YFK132 was isolated from S. cerevisiae strain YFK007 (wild-type; MY2095; ATCC 74060) by standard ethylmethane-sulfonate (EMS) mutagenesis procedures (Sherman et al., 1986 in "Laboratory course manual for methods in yeast genetics", Cold Spring Harbor Press). Parental strain YFK007 is sensitive to about 50 mcg/ml

of FK506 and is insensitive to 100 mcg/ml CsA. Mutant strain YFK132 is hypersensitive to FK506.

YFK007 was grown overnight in 25 ml of YEPD at 30°C. The cells were harvested by centrifugation, and resuspended in 10 ml of 0.05 M potassium phosphate buffer (pH 7) at a density of 3×10^8 cells/ml. The cell suspension was diluted to 1.24×10^8 cells/ml and divided into two samples

To one sample, 0.588 ml of EMS (Sigma Cat. No. M0880) was added. The same volume of distilled water was added to the second sample as a control. Treated cell suspensions were incubated at 25°C. At various times, samples were removed and added to 8 ml of 5% sodium thiosulfate to quench the mutagenesis. Quenched cells were diluted in water, plated on YEPD agar and incubated at 25°C. Cells from EMS-treated and untreated cultures were spread on YEPD plates at various dilutions, and colonies were counted to determine cell viability after the mutagenesis.

YEPD plates containing mutagenized colonies were replica plated onto SC agar containing 0, 1 or 10 mcg/ml of FK506, and incubated at 25°C. Approximately 1,200 colonies were screened. Three cultures that failed to grow on SC medium + FK506 were identified and analyzed further.

One of these cultures, designated YFK132, exhibited an FK506-hypersensitive phenotype (sensitive to 0.1 mcg/ml FK506), was sensitive to 10 mcg/ml CsA, and was slow growing.

EXAMPLE 4

Backcrossing YFK132, an fks1-1 mutant

To determine whether the phenotypes of YFK132 were the result of a single mutation, tetrad analyses were performed on crosses between mutant and wild-type strains.

YFK132 was crossed to the wild type strain YFK005 and a meiotic segregant from the resulting diploid backcrossed to YFK007 two times to generate strains YFK531-5A, YFK532-7C, and

YFK532-10B. The FK506 hypersensitive and slow growth phenotypes of YFK132 cosegregated in all crosses, indicating that these phenotypes resulted from a mutation in a single gene. YFK132 is an fks1-1 mutant of YFK007.

EXAMPLE 5

Testing the echinocandin sensitivity of YFK132

The sensitivity of YFK132 to the echinocandin L-688,786 was determined in a disc-diffusion assay.

YFK132 and its parent (YFK007) were grown in 2.5 ml of liquid SC medium and diluted to 6.25 x 10^7 cells/ml with distilled water. Molten SC medium containing 2% agar (130 ml) was inoculated with 4 ml of diluted culture and immediately poured into 245 x 245 mm bioassay plates. After the medium had solidified, sterile filter discs containing FK506 (1, 10 and 50 mcg) or L-688,786 (1, 10 and 50 mcg) were placed on the surface of the medium and incubated at 28°C. After 18 hours, zones of growth inhibition were measured.

As shown in the following table, YFK132 is more sensitive to L-688,786 than its parent strain (YFK007).

Amount of L-688,786/disc (micrograms)	Zone Sizes (mm) YFK007	YFK132
1	0	8.4
10	8.7	16.8
50	8.7	18.0

EXAMPLE 6

Cloning of FKS1 by complementation of fks1-1

A. General approach

The 1,3-beta-D-glucan synthase gene (FKS1) was cloned by complementation of the FK506 hypersensitive phenotype of YFK532-10B (MATa, ade2-101, his3-Δ200, leu2-Δ1, lys2-801, trp1-Δ1, ura3-52, fks1-1). The general approach to cloning genes by complementation of mutant phenotypes is outlined by M. D. Rose and J. R. Broach (in GYG, pp. 195-230).

Library plasmid DNA was obtained from E. coli ATCC 37415 . This library was created by M.D. Rose, et al., (Gene, 60, 237-243, 1987), by inserting 10- to 20-kb Sau3AI partial-digest fragments of yeast genomic DNA from strain GRF88 into the yeast shuttle vector YCp50.

B. Preparation of electroporatable cells

Cells of YFK532-10B were prepared for transformation by electroporation, essentially as described by D.M. Becker and L. Guarente, (in Guthrie and Fink, supra, pp. 182-187). Recipient cells were grown on agar plates containing YPAG medium supplemented with 0.004% adenine sulfate. Cells from a fully grown patch (1 mm X 5 mm) were inoculated into 50 ml of YPAD-25C medium (YPAD supplemented with 25 mM $CaCl_2$) in a 250 ml Erlenmeyer flask and incubated at 30°C on a rotary shaker (225 rpm, 2" throw). The culture was grown to an optical density of 1.3 at 600 nm and transferred to a sterile 50-ml disposable polypropylene centrifuge tube. Cells were harvested by centrifugation at 3500 rpm for 5 min at 4°C in a Sorvall RT6000 refrigerated centrifuge. The cell pellet was resuspended with 25 ml ice-cold sterile water by vortexing at full speed, harvested by centrifugation and washed again with 25 ml ice-cold sterile water. The cell pellet was resuspended with 10 ml ice-cold sterile 1 M sorbitol. The washed cell suspension was transferred to a sterile 10-ml disposable

polypropylene centrifuge tube, and the cells were harvested by centrifugation at 3500 rpm for 10 min at 4°C. The cell pellet was resuspended with 0.1 ml ice-cold sterile 1 M sorbitol.

C. Electroporation of recipient cells

A portion (50 mcL) of the washed cell suspension was transferred to a sterile microfuge tube. An aliquot (1 mcL containing ca. 500 ng) of library plasmid DNA (Bank A) was added to the cells, mixed gently, and incubated on ice for about 5 min. The cell suspension was transferred to a cold 0.2-cm sterile electroporation cuvet and pulsed at 1.5 kV, 25 uF, 200 ohm (BioRad Gene Pulser with Pulse Controller). Immediately 3 ml ice-cold sterile 1 M sorbitol was added and mixed gently.

Fifteen aliquots (0.2 ml) were transferred to sterile culture tubes. Uracil drop-out/sorbitol soft agar (3.5 ml) containing 1 M sorbitol in soft (0.6%) agar at 46°C was added to each tube to form a mixture, and each mixture poured over a 2% agar plate made with the same sorbitol-containing medium, giving a total of fifteen plates. The procedure was repeated until 210 plates were obtained.

The plates were incubated at 30°C. After 24 hr each plate was overlayered with 3 ml of uracil drop-out/sorbitol soft agar containing 1 mcg/ml FK506 (a 5 mg/ml stock solution of FK506 in ethanol was added to autoclaved medium that had been cooled to 55°C). The plates were incubated at 30°C for 6 more days. Cells from transformant colonies were purified by streaking for single colonies on agar plates containing uracil drop-out medium supplemented with 0.1 mcg/ml FK506.

D. Isolation of plasmid pFF119

Colonies of the purified transformants were inoculated into 1.5 ml uracil dropout medium in 16-mm culture tubes and incubated in a tube roller at 30°C for two days. Plasmid DNA was prepared essentially as described by J.N. Strathern and D.R. Higgins (in Guthrie and Fink, supra, pp. 319-329) according to Method 1 and transformed

into competent E. coli strain DH11S (Bethesda Research Laboratories). Plasmid DNA was prepared from ampicillin-resistant E. coli using the QIAGEN-tip 500 procedure (QIAGEN Inc., Chatsworth, CA). The resulting plasmid was designated pFF119.

The ability of pFF119 to complement the fks1-1 mutation was confirmed by spontaneous curing of the plasmid in the original transformant. Curing restored the FK506 hypersensitive phenotype. Retransformation with pFF119 restored FK506 resistance.

E. Localization of the fks1-1 Complementing DNA

pFF119 was digested with various combinations of restriction endonucleases and analyzed by agarose gel electrophoresis. The results showed that pFF119 contained an insert of about 15 kb of DNA.

An 11-kb SphI fragment from within the 15-kb region was transferred to the SphI site of plasmid YCplac33 [R.D. Gietz and A. Sugino, Gene, 74:527-534 (1988)] in both orientations giving plasmids pFF133 and pFF135. These plasmids were also capable of complementing the FK506 hypersensitive phenotype of the fks1-1 mutation.

Nested subclones of the cloned DNA were created by linearizing pFF133 and pFF135 with BamHI, digesting partially with Sau3AI, and recircularizing the molecules with DNA ligase. Only two of the subclones (pFF172 and pFF173) were capable of complementing fks1-1. The complementing DNA was thus localized to a region with a minimum of 6.0 kb and a maximum 7.8 kb of DNA, between the first SphI site and the second BglII site.

F. Identification of the fks1-1 Complementing DNA as FKS1

An insertion-deletion allele of the fks1-1 complementing DNA was created in the following manner. The 8.8-kb SphI-PstI fragment of pFF133 was inserted between the SphI and PstI sites of the polylinker of the E. coli vector pGEM-5Zf(+) (Promega, Madison, WI) giving plasmid pFF174. The 1.3-kb BamHI-XhoI HIS3 fragment from

plasmid pJJ215 (J.S. Jones and L. Prakash, Yeast, 6:363-366 (1990)) was inserted by blunt-end ligation (see Current Protocols, p. 3.5.10) between the two KpnI sites of plasmid pFF174 giving plasmids pFF186 (sense orientation) and pFF187 (antisense orientation). The 6.6-kb insertion-deletion fragments were excised by digestion with SstI + SphI and purified by agarose gel electrophoresis. The insertion-deletion mutation was created by one-step gene replacement (see R. Rothstein, GYG, pp. 281-301). This disruption was confirmed by Southern blot hybridization analysis of genomic DNA digested with PstI and probed with the 8.8-kb SphI-PstI fragment from plasmid pFF174. The undisrupted parent gives a single 9.8-kb genomic fragment which hybridizes with the probe. A disruption mutant in which HIS3 is inserted in the sense orientation, for example YFF2409, gives 3.9- and 3.7-kb fragments, while an antisense disruption mutant, for example YFF2411, gives 4.9- and 2.7-kb fragments. A haploid strain with the insertion-deletion allele has phenotypes essentially identical to an fks1-1 mutant: it is slow-growing, hypersensitive to FK506, and hypersensitive to L-733,560. Diploids created by crossing insertion-deletion haploids with fks1-1 haploids are slow-growing and hypersensitive to FK506 showing that the insertion-deletion mutation fails to complement the fks1-1 mutation.

These results prove that the two alleles are in the same gene and that pFF119 carries the FKS1 gene. The insertion-deletion mutations are therefore referred to as fks1-5::HIS3 and fks1-6::HIS3.

EXAMPLE 7

Other Strains of S. cerevisiae Possess Variants of FKS1

Southern hybridization analysis of genomic DNA isolated from various strains of S. cerevisiae and digested with different restriction enzymes revealed that some strains have a variant of FKS1 which has a restriction map which differs slightly from that for the gene in GRF88. Strains with FKS1 genes with restriction maps like that for GRF88 (G.R. Fink) include SC347 (J. Hopper), W303-1B (R.

Rothstein), S288C (R.K. Mortimer), and A384A (L. Hartwell). Strains with ones like that for YFK007 include YPH1 (Phil Hieter), YFK005, YFK093, DS94 (E. Craig), and DS95 (E. Craig).

EXAMPLE 8

Isolation of FKS2 by cross-hybridization with FKS1 DNA.

A 2.5-kb PstI genomic fragment crosshybridizing with the FKS1 probe was detected in Southern blots of genomic DNA from S. cerevisiae. This fragment was not derived from the FKS1 region of the genome. When genomic DNA was digested with BglII + PstI the fragment was 1.7 kb in size. Genomic DNA was isolated from strain YFK007, digested with BglII + PstI, and fractionated on an agarose gel. The region of the gel containing the crosshybridizing fragment was excised, and DNA was isolated using the QIAEX extraction procedure (QIAGEN Inc.). The extracted DNA was inserted between sites for BamHI and PstI in the polylinker of the plasmid pGEM-3Zf(+), and the ligated DNA was transformed into strain DH11S (Bethesda Research Laboratories). Ampicillin resistant transformants were screened for the presence of the crosshybridizing DNA by colony hybridization (Maniatis, supra). Plasmid DNA was isolated from positive clones and digested with KpnI + PstI. KpnI was used in place of BglII, since the BglII site was lost during the ligation with the vector. The presence of a 1.7-kb fragment crosshybridizing with the FKS1 probe was confirmed by Southern blot hybridization analysis. The resulting plasmid is called pFF250.

The 1.7-kb fragment was used to screen a lambda library (Stratagene, cat. no. 951901) of yeast genomic DNA from strain S288C by plaque hybridization (Maniatis, supra). DNA was isolated from positive clones, digested with various restriction enzymes, and analyzed for hybridizing fragments by Southern blot hybridization. A 10-kb EcoRI fragment carrying the hybridizing region was cloned into the EcoRI site of pBluescript II KS(+) (Stratagene) in both orientations giving plasmids pFF334 and pFF336.

An insertion-deletion mutation of the 1.7-kb BglII-PstI DNA was created by inserting the 0.8-kb PstI TRP1 fragment from pJJ246 (J.S. Jones and L. Prakash, Yeast, 6:363-366 (1990)) between the AflII and BbsI sites by blunt end ligation. The 2.1-kb disruption fragment was excised with PstI + KpnI. The insertion-deletion mutation was inserted by one-step gene replacement into the chromosome of a heterozygous fks1-5::HIS3/+ and homozygous trp1/trp1 diploid. Genomic DNA from Trp^+ transformants was digested with BglII + HindIII + PstI. The undisrupted locus gives a 1.7-kb hybridizing fragment, while the insertion-deletion mutation gives 1.4- and 0.7-kb fragments.

A transformant heterozygous at the locus of the insertion-deletion mutation was sporulated and dissected on YPAD. Trp^+ His^- spores were viable. However, Trp^+ His^+ spores were inviable. The insertion-deletion mutation thus defines a new locus FKS2, and the insertion-deletion mutation of this locus (fks2::TRP1) is synthetically lethal with fks1-5::HIS3. These results are interpreted to mean that the products of FKS1 and FKS2 have overlapping functions and that when the function of each is inactivated, either through genetic disruption or by inhibition of their gene products with L-733,560, cells are not viable.

EXAMPLE 9

Construction of a plasmid DNA library containing the FKS1gene

A genomic DNA library containing the FKS1 gene was constructed in the plasmid YEp24 by standard methods (Rose and Broach, 1991, Methods in Enzymology, 194:195-230).

High molecular weight genomic DNA was prepared from yeast strain YFK093 (MY2088, ATCC 74055), partially digested with Sau3AI and size-fractionated over a sucrose gradient. A fraction of Sau3AI-digested DNA ranging from 10-15 kb was partially-filled in with Klenow fragment of DNA polymerase I using dATP and dGTP.

The multicopy vector (YEp24) was digested with SalI, and partially-filled in with Klenow fragment of DNA polymerase I using dCTP and dTTP. After the fill-in reactions, the DNAs were phenol extracted once and ethanol precipitated. Partially-filled in genomic and vector DNAs were ligated and transformed into HB101 cells by selecting for ampicillin resistance.

Two independent libraries were generated by pooling clones generated by separate transformations. One library contained ca. 34,100 transformants while the second library contained ca. 15,000 transformants. The frequency of recombinants in these libraries was judged to be ~95% by restriction enzyme digestions.

EXAMPLE 10

Isolation of R560-1C, a mutant of S. cerevisiae resistant to L-733,560

Strain W303-1A was transformed by the spheroplast method (MYG) with yeast genomic libraries obtained from D. Botstein (1982. Cell, 28:145-154). Transformants selected on uracil dropout medium were pooled and stored at -80°C in 20% glycerol. After determining the titer (colony forming units(CFU)/ml), aliquots of the stocks were spread at $\approx 5 \times 10^3$ CFU per plate onto uracil dropout medium containing the semisynthetic echinocandin L-733,560 at 0.5 mcg/ml or 1.25 mcg/ml. This concentration of L-733,560 is sufficient to select for resistant clones. Twenty-seven drug resistant colonies were isolated. The resistance phenotype of these clones was quantitated in a liquid MIC assay. Briefly, L-733,560 was serially diluted across the wells of a sterile microtiter plate such that the concentration in each row ranged from 16 to 0.03 mcg/ml in 2-fold increments after an equal volume of a cell suspension in liquid uracil dropout medium was added. After 24 h at 30°C, plates were read in a spectrophotometer at a wavelength of 600 nm, and the percent of control growth in each well (relative to a no-drug control well) was calculated. The resulting dose-response curve was used to determine the resistance relative to the parent strain. One clone was 16-32-fold more resistant than the parent

strain; the others were 2 to 4 - fold more resistant. The most resistant clone, R560-1, was characterized further.

Because this strain was isolated as a transformant from the genomic library, it was expected that resistance would be due to the gene residing on the plasmid contained in R560-1. To test this, the strain was cured of the plasmid by selection with the 5-fluoroorotic acid method (MYG). Loss of the plasmid and its resident URA3 gene renders cells resistant to 5-fluoroorotic acid, and the uracil auxotrophy was confirmed by the absence of growth on uracil dropout medium. Surprisingly, the drug resistance of the cured derivative (R560-1C) was unchanged by the loss of the plasmid. This results suggests that R560-1C is a spontaneous echinocandin-resistant mutant of strain W303-1A. R560-1C was challenged with other beta-glucan synthase inhibitors such as L-688,786, L-646,991 (cilofungin), and L-687,781 (papulacandin) in a liquid MIC assay. The results illustrate that the resistance phenotype is not specific to L-733,560 but also includes structurally related and unrelated inhibitors of 1,3-beta-D beta-glucan synthesis. To determine whether the phenotypes of R560-1C were the result of a single mutation, tetrad analyses were performed on diploids formed by crossing mutant and wild-type strains. R560-1C was mated to the wild type strain W303-1B, sporulated, dissected, and the sensitivity to L-733,560 quantitated by liquid MIC assay. Drug resistance segregated 2:2 in these tetrads, proving that resistance is due to a mutation in a single gene. This mutation is called fks1-2.

EXAMPLE 11

Cloning the FKS1 gene by complementation of the fks1-2 mutation in R560-1C

Using information from the genetic analysis of R560-1C, a screen was devised to clone the wild type allele of fks1-2. When R560-1C was mated to the wild type strain W303-1B, the resulting heterozygous diploids were intermediate in sensitivity to L-733,560, suggesting that a single copy of the wild type FKS1 gene would make

the mutant more sensitive to echinocandins. By cloning a library of S. cerevisiae DNA into R560-1C, transformants could be screened for plasmid-dependent intermediate sensitivity to L-733,560. Broth microdilution and replica plating methods discriminated between heterozygous diploids and R560-1C at 4 mcg/ml L-733,560.

The S. cerevisiae total genomic library of Example 9 was transformed into S. cerevisiae R560-1C by the spheroplast method (Maniatis, supra). Ura^+ clones were selected on uracil dropout medium, scraped from the plates, pooled, and stored frozen at -80°C in 20% glycerol. Aliquots of the library were plated onto uracil dropout medium at 200-300 CFU/plate. After incubation at 30°C for 24 h, the colonies on each plate were replica plated to: 1) uracil dropout medium plates supplemented with 4 mcg/ml L-733,560; and 2) uracil dropout medium plates. Putative clones were identified by poor growth on the drug supplemented plate and strong growth on the drug-free plate. Three additional tests were used to establish which potential clones were truly drug sensitive. In one test, putative clones were inoculated into liquid uracil dropout medium in mirotiter plates and grown for 24 h at 30°C. Using a Dynatech inoculator, cells from each well were inoculated into: 1) uracil dropout medium supplemented with 4 mcg/ml L-733,560; and 2) uracil dropout medium. Growth was quantitated spectrophotometrically, and poor growth in drug supplemented medium was scored as positive. In a second test for drug resistance, colonies were patched directly to uracil dropout medium plates supplemented with 4 mcg/ml L-733,560 and scored for poor growth after 24 h at 30°C. In the third assay, putative clones were patched to uracil dropout medium plates, grown for 24 h at 30°C, then replica plated to uracil dropout medium supplemented with 10mcg/ml L-733,560. Growth was scored after 24 h at 30°C.

Nine putative clones were positive in all assays for intermediate drug sensitivity. One strain (S277) was nearly as sensitive to L-733,560 as the wild type strain. To quantitate the drug sensitivity of clone S277, a liquid MIC assay was performed. The drug-sensitive clone (S277) was significantly more sensitive than the mutant

(R560-1C), and nearly as sensitive as the wild type strain (W303-1A). To verify that the intermediate drug sensitivity of S277 was due to the cloned gene it contained, the plasmid was cured by the 5-fluoroorotic acid method. An MIC assay revealed that loss of the plasmid resulted in a reversal of the intermediate sensitivity to L-733,560, such that the plasmid-cured clone was as resistant to drug as the original resistant mutant (R560-1C). Finally, retransforming R560-1C with plasmid DNA isolated from S277 yielded Ura^+ clones which were identical to the original drug sensitive clone (S277) in their intermediate sensitivity to L-733,560.

Plasmid DNA was isolated from the drug sensitive clone (S277) and transformed into E. coli by methods described in Maniatis. Two plasmids with different size inserts were isolated and characterized by restriction endonuclease mapping; one (pJAM53) had a 14 kb insert, and the second (pJAM54) had an 8 kb insert. Restriction mapping illustrated that the insert in pJAM54 was entirely contained within the 14kb fragment of pJAM53. Both plasmids conferred intermediate sensitivity to L-733,560, as judged by liquid MIC assays, when they were introduced by transformation into strain R560-1C.

EXAMPLE 12

Overexpression of calcineurin in the fks1-1 mutant

Individual phage clones containing the calcineurin genes were identified from a yeast genomic DNA library of strain S288C in lambda-DASH (Stratagene, cat. no. 943901) by hybridization to probes synthesized from yeast genomic DNA by PCR (Foor et al., Nature, 360:682-684 (1992)). The CNA2 and CNB1 genes were mapped to 4.3-kb BglII and 1.3-kb EcoRV DNA fragments within isolated phage clones, respectively. The CNB1 fragment was inserted into the SmaI site of pBluescript II KS(+) in the lacZ orientation and transferred as a BamHI-EcoRI fragment to the TRP1-selectable multicopy yeast shuttle vector YEplac112 (Gietz & Sugino, 1988, supra), giving plasmid YEp-B. The CNA2 fragment was inserted into the BamHI site of

YEp-B giving YEp-A2-B. This plasmid was transformed by electroporation into the fks1-1 strain YFK531-5A giving strain YFK798.

EXAMPLE 13

A. Use of YFK532-7C, an fks1-1 mutant strain, for screening for glucan synthase inhibitors

Strain YFK532-7C, an fks1-1 mutant, is at least a thousand fold more sensitive to FK506 and CsA (known calcineurin inhibitors) than is strain YFK007, an FKS1 wild-type strain. YFK007 and YFK532-7C can be used to screen for calcineurin inhibitors.

Strain YFK532-7C is also 8-10 fold more sensitive than strain YFK007 to glucan synthase inhibitors of both the echinocandin and papulacandin classes. Therefore, these strains can be used to screen for glucan synthase inhibitors.

Counter screening strains were devised for identifying calcineurin inhibitors. Overexpression of yeast calcineurin in the fks1-1 mutant (strain YFK798) constitutes the most general of these. Any inhibitor targetting calcineurin shows either zone diameter reduction or a decrease in zone clarity (sometimes both). Glucan synthase inhibitors show neither effect and thus can be distinguished from calcineurin inhibitors.

For positively identifying glucan synthase inhibitors, screening with strain R560-1C and its parent W303-1A was instituted in a manner identical to that described for calcineurin inhibitors. Complete loss of a zone, or marked reduction in size, on R560-1C versus W303-1A indicates a glucan synthase inhibitor. No zones are seen with calcineurin inhibitors using this pair of strains.

B. Description of the laboratory procedure

The initial screen consists of a two-plate differential zone size determination comparing the sensitivity of fks1-1 yeast mutant (YFK532-7C, hypersensitive to FK506 and CsA) to that of the FKS1

wild-type strain (YFK007). Each strain is grown at 28-30°C in YPAD/10 mM $CaCl_2$ medium with shaking at 220 rpm (to mid or late log phase). The cultures are diluted 1:10 with water, and the OD values of the dilutions are measured at 600 nm (against a blank of YPAD/10 mM $CaCl_2$ similarly diluted 1:10 in water). The OD value is multiplied by 10 to estimate the OD of the culture. Portions (100 ml) of YPAD/10mM $CaCl_2$ medium containing 1.5% agar, equilibrated at 45°C in a water bath, are seeded with culture such that the final cell density in the agar would have an OD value of 0.015 (i.e., 3 x 10^6 cfu/ml; a sample calculation is provided below). The seeded agar is poured into 500 cm^2 Nunc plates. Once the agar plates have solidified, 10 mcL aliquots of samples containing test compounds, such as fermentation extracts, are dissolved in water, 100% methanol or ethanol, or up to 50% DMSO and are placed on each member of the two-plate set in 11 by 8 arrays.

The plates are incubated for 48 h at 28-30°C. Diameters of the zones are read to the outermost edge and recorded in mm. The clarity of the zone is reported as clear (no designation), hazy (h), or very hazy (vh). Very hazy zones are best seen by viewing the plate under an elevated light placed between the assay dish and a dark wall.

STANDARDS

1. L-679,934 (FK506) Dissolve in methanol.
2. L-644,588 (cyclosporin A) (Sandimmune) is sold in a Cremaphor vehicle at a concentration of 100 mg/ml. Dilutions may be made in 50% methanol or 50% ethanol with vigorous mixing at each step. The cremaphor remains very cloudy in these dilutions but the cyclosporin is bioavailable.
3. L-733,560 Dissolve in methanol.
4. L-687,781 (dihydropapulacandin) Dissolve in methanol.
5. L-636,947 (aculeacin) Dissolve in methanol.

Store all standards at -20°C.

Sample calculation of inoculum dilution

Overnight yeast cultures will have OD values ranging from 7 to 10 (i.e., 0.7 to 1.0 for 1:10 dilutions). A culture with an OD of 8.9 is diluted 1:593 to give a suspension with an OD of 0.015. Therefore, a 100-ml portion of YPAD/10 mM $CaCl_2$ agar would be inoculated with 169 ml of culture.

Primary Screen

Controls	Zone size (mm)		
	YFK007 (wild type)	YFK532-7C (fks1-1)	YFK798 (fks1-1 + CN)
20 ng FK506	none	15-17	13 vh edges
10 mcg CsA	none	18-20	15 vh edges
10 mcg L733,560	16-18	22-24	22-24
20 mcg aculeacin	22-24	27-30	27-30
20 mcg papulacandin	20-22	26-28	26-28

Secondary Screen

Controls	Zone size (mm)	
	W303-1A	R560-1C
20 ng FK506	none	none
10 mcg CsA	none	none
10 mcg L733,560	16-18	8 vh
20 mcg aculeacin	18-22	17-20
20 mcg papulacandin	15-18	14-16

Results in primary screen

A zone at least 2 mm larger on YFK532-7C than YFK007 indicates the presence of either a calcineurin or glucan synthase inhibitor.

A zone that is reduced or hazier on YFK798 compared that seen on YFK532-7C indicates that the unknown is a calcineurin inhibitor.

A zone that is reduced on R560-1C compared with W303-1A indicates that a glucan synthase inhibitor is present.

EXAMPLE 14

Glucan Synthase Assay

Cell free extracts were prepared from mutant and wild type cells grown to logarithmic phase as previously described (Kang and Cabib, PNAS, 83:5808-5812, 1986). After homogenization with glass beads, the unbroken cells and debris were removed by a low speed centrifugation (1,000 x g for 5 min). The supernatant fluids were centrifuged at 100,000 x g for 60 min and the pellets were washed with 2.5 ml (per gram of wet cells) of buffer containing 0.05 M potassium phosphate (pH 7.5), 0.5 mM DTT, and 1.0 mM PMSF. The washed pellet was resuspended in the same buffer containing 5% glycerol. This served as the microsomal membranes source containing both chitin and glucan synthase enzymatic activities. The standard 1,3-beta-D glucan synthase reaction was initiated by mixing 35 mcg protein in cocktail I, which included TEK buffer (125 mM Tris chloride, pH 7.5, 31 mM KF, and 1 mM EDTA), 25% PBS, pH 7.0, 3.31 mcM GTP-gamma-S, and 0.25% BSA in a total volume of 69 mcL, with cocktail II, which included 4 units alpha-amylase, 25 mcg UDP-glucose, and 1 microCi UDP-^{3}H-glucose, in a total volume of 11 mcL. Following 150 minutes of incubation at 30°C, the incorporation of UDP-^{14}C-glucose into glucan was measured after precipitation with trichloroacetic acid.

EXAMPLE 15

Chitin synthase assay

125 mcg of the above extracts were trypsin activated and mixed with an equal volume (50 mcL) chitin synthase reaction cocktail, which included 0.5 M Tris, pH 7.5, 40 mM MgCl, 320 microM GlcNAc, ^{14}C-UDP-GlcNAc substrate mix, and 0.8% digitonin. After 30 minutes of incubation at 30°C, the incorporated ^{14}C-glucose was

precipitated with 10% trichloroacetic acid, collected onto Whatman glass microfiber GF/A disks and counted.

EXAMPLE 16

Isolation of the echinocandin-resistant mutants MS10 (MY2144) and MS14 (MY2145)

MS10 and MS14, were isolated as echinocandin resistant mutants in two different experiments.

In the first experiment, approximately 45 mcg (40 mcL of 1.12 mcg/ml solution) of the semisynthetic echinocandin L-733,560 was spread over the surface of each of four plates containing YNBD solid medium (YNBD medium is the same as the SC medium but lacking amino acids). The solution was allowed to air-dry before 1 x 10^6 cells of the S. cerevisiae strain X2180-1A freshly grown overnight on YNBD broth was plated onto each plate. Following growth at 28°C for four days, three colonies capable of growth in presence of L-733,560 were picked as echinocandin-resistant mutants. One of those mutants was designated MS14.

The second experiment was performed as described above with the following modifications: The concentration of L-733,560 used was approximately 22.5 mcg/plate. The inhibitor was added to 20 ml of YNBD media that had been melted and then cooled to 50°C. Four plates prepared; then 1 x 10^6 cells of S. cerevisiae strain X2180-1A was spread over the surface of each plate. Following growth at 28°C for 4 days, 12 resistant colonies were isolated. One of those mutants was designated MS10.

Based on these experiments the mutation frequency of the mutant MS14 is 1.3×10^{-6}, while the mutation frequency of the mutant MS10 is 3×10^{-6}.

EXAMPLE 17

Characterization of MS10 and MS14 Mutants

MS10 and MS14 did not exhibit multiple drug resistance when tested against a panel of more than 30 inhibitors affecting cell wall, membrane, sterol, and protein synthesis. Cells of the yeast strains MY2144 and MY 2145 carrying the respective MS10 and MS14 mutations were grown overnight in YPAD and SC media. From the overnight cultures, cells were diluted 1:10 in the same media and allowed to further grow for 4-6 hrs. The drug resistance/sensitivity tests were conducted by the disc diffusion assay on plates containing 20 ml of solid YPAD or SC media and 3 x 10^6 cells. The cells were added to premelted media that was cooled to 50°C before pouring onto plates. Sterile filter discs containing different drugs were placed on the surface of the plates followed by incubation at 28°C for 1-2 days. Sizes of the zones of growth inhibition were measured as an indication of relative drug resistance/sensitivity. The MS14 mutant is supersensitive to the chitin synthesis inhibitor nikkomycin Z and resistant to the echinocandin L-733-560.

The dominance/recessiveness relationships of the mutations in MS10 and MS14 were determined by comparing the drug resistance phenotype of haploid and diploid cells using both the disc diffusion and the broth microdilution assays. The results of those assays show that the nikkomycin Z-supersensitivity of the MS14 cells is recessive while the echinocandin-resistance phenotype is semi-dominant. In contrast, the echinocandin-resistance phenotype of the MS10 cells is dominant .

The data in the following table are the minimum concentration of the various drugs required to inhibit the growth of each the mutants and their parent X2180-1A.

Strain	MIC L-733,560 (uM)	Papulacandin (mcg/ml)	Nikkomycin Z (mcg/ml)
X2180-1A	0.045	5.5	>100
MY2144 (fks 1-3)	0.75	15	>100
MY2145	2.0	5.5	0.2

EXAMPLE 18

Mutant glucan and chitin synthesis enzymatic activities

Crude enzyme preparations associated with cell membranes were tested for glucan and chitin synthesis activities. The sensitivity of the mutant 1,3-beta-D glucan synthase to L-733,560 and papulacandin was tested along with the sensitivity of the chitin synthases to nikkomycin Z.

Results of these experiments revealed that both MS10 and MS14 have normal levels of 1,3-beta-D glucan synthase that are highly resistant to L-733,560 but only marginally resistant to papulacandin. The chitin synthase is not affected in its sensitivity to nikkomycin Z. The data in the following table show the IC_{50}s for the glucan synthase inhibitors (L-733,560 and papulacandin) and the chitin synthase inhibitor (nikkomycin Z) in 1,3-beta-D glucan synthase and chitin synthase assays, respectively. Equal amounts of membrane proteins were used to prime each reaction.

Strain	IC_{50} L-733,560 (uM)	Papulacandin (mcg/ml)	Nikkomycin Z (mcg/ml)
X2180-1A	6.1	5.08	0.74
MY2144 (fks 1-3)	38.0	11.1	0.60
MY2145 (fks 1-4)	65.0	11.5	0.64

EXAMPLE 19

Cloning of a gene complementing nikkomycin Z supersensitivity.

A genetic cross was set up between MS14 (echinocandin-resistant and nikkomycin Z-sensitive) and the wild-type strain GG100-14D (echinocandin-sensitive and nikkomycin Z-resistant). The resultant diploid cells were sporulated and tetrads were dissected followed by phenotypic and drug resistance analysis of the meiotic segregants. The results demonstrated that the two phenotypes of echinocandin-resistance and nikkomycin Z supersensitivity co-segregate, suggesting a single gene mutation is responsible for the two phenotypes.

The strain D1-22C is a meiotic segregant from the above cross. This strain is echinocandin-resistant, nikkomycin Z-supersensitive and Ura$^-$. Cells of strain D1-22C were transformed with the yeast DNA genomic library constructed in the centromere-based vector YCp50 (M. Rose et al., Gene, 60:237-243, 1987). This is the same DNA library that was used in Example 6. Double selection for uracil-prototrophy and nikkomycin Z-supersensitivity was conducted by plating the transformants on Ura dropout plates containing 75 mcg/ml of nikkomycin Z. Only colonies that can grow in absence of uracil and in the presence of nikkomycin Z will grow. Hence, this assay selected for transformants that have received the recombinant plasmids carrying DNA fragments capable of complementing the nikkomycin Z supersensitivity phenotype. Out of 20 uracil-prototrophic nikkomycin Z-resistant colonies isolated by this scheme, 3 clones were also sensitive to the echinocandin L-733,560. One of those three transformants is the strain designated 9-3B an contains a plasmid with the complementing gene. The plasmid in this strain was designated pMS14 since it complements the MS14 phenotypes in the transformed mutant cells (strain D1-22C). The pMS14 plasmid was rescued from the yeast cells (clone 9-3B), propagated in E. coli and retransformed into strain D1-22C. Three transformants were tested for resistance/sensitivity to L-733,560 and nikkomycin Z by the broth

microdilution assay. In the 3 transformants tested, the echinocandin-resistance and the nikkomycin Z sensitivity, were reversed.

EXAMPLE 20

Allelism relationship between the mutations in MS10 and MS14

A uracil auxotroph carrying the echinocandin-resistance mutation from MS10 was constructed by crossing MS10 with GG100-14D. An echinocandin-resistant meiotic segregant was transformed with the single copy recombinant plasmid pMS14 and transformants were tested for susceptibility to echinocandins by the broth microdilution assay. All three transformants tested showed sensitivity to L-733,560. In contrast, mutant cells transformed with the control plasmid YCp50 remained echinocandin-resistant. Thus, the recombinant plasmid pMS14, complementing both the echinocandin resistance and nikkomycin sensitivity phenotypes of the mutation from MS14 also complements the echinocandin resistant phenotype of MS10. This result suggests that the two mutations represent two different alleles of the same gene.

EXAMPLE 21

A. pJAM54 complements the mutations from MS10 and MS14

Yeast cells containing the mutations from either MS10 or MS14 were transformed with the multiple copy plasmid pJAM54 (containing FKS1). Like pMS14, pJAM54 complemented the two phenotypes of echinocandin resistance and nikkomycin sensitivity caused by the mutation from MS14. pJAM54 also complements the echinocandin resistance phenotype of strain MS10.

B. Cross-hybridization between pMS14 and pJAM54

The plasmid pJAM54 (a multicopy plasmid containing FKS1) and the single copy plasmid pMS14 were digested with restriction enzymes and analyzed by Southern hybridization analysis

using an FKS1 internal fragment as a hybridization probe. Both Southern and restriction enzyme analysis showed that pJAM54 and pMS14 contain the same gene, namely FKS1. The mutation in MS10 is therefore referred to as fks1-3, and the mutation in MS14 is referred to as fks1-4.

EXAMPLE 22

Isolation of FKS1 and FKS2 homologs from Cryptococcus neoformans

To determine whether FKS1 homologs exist in the C. neoformans B-3502 chromosome, a sample of total genomic DNA from this strain was digested with HindIII, and fragments were separated on a 0.8% agarose gel. The gel was probed with the AflII-XhoI fragment from pJAM54 by the method of Southern, and washed under high stringency conditions. A fragment approximately 15 kb in length was visible on the autoradiogram. Most likely, this fragment contains all or a portion of the FKS1 homolog in C. neoformans B-3502.

Similar Southern blot hybridization experiments are carried out with an FKS2 fragment as the probe.

A phagemid cDNA library of poly (A)+ RNA from C. neoformans B-3502 is constructed essentially according to the method of Edman et al., (1990. Mol. Cell Biol., 10(9):4538-4544). E. coli XL-1B is co-infected with the phagemid library and a helper phage (R408) such that approximately 500 plaques are formed per agar plate. Plaques are lifted to nitrocellulose and probed by standard methods, using a fragment of FKS1 as a probe. After washing, filters are exposed to film, and the autoradiograph is used to identify specific phagemid clones which hybridize with FKS1. Plasmid DNA is then isolated from the cDNA transfectants, propagated, and analyzed by digestion with restriction endonucleases.

To isolate FKS2 homologs similar experiments are carried out with an FKS2 probe.

EXAMPLE 23

Isolation of FKS1 and FKS2 homologs from Pneumocystis carinii

Whole rat lungs from P. carinii-infected male Sprague-Dawley rats are homogenized with a Brinkmann homogenizer and DNA is isolated as described (P.A. Liberator, et al., 1992. J. Clin. Micro., 30(11): 2968-2974). Two to five micrograms of purified DNA are digested with a restriction endonuclease such as EcoRI, and the fragments are separated on an agarose gel. DNA is transferred to a solid support such as nitrocellulose and probed by the method of Southern (Southern, E.M. 1975. J. Mol. Biol., 98:503-517) for fragments with homology to FKS1. By washing the blot at a reduced stringency, weakly homologous genes can be identified.

Similar Southern blot hybridization experiments are carried out with an FKS2 fragment as the probe.

The P. carinii FKS1 homologs are cloned by preparing a mini-library from the region of the agarose gel where the hybridizing fragment was visualized on the Southern blot. Following phenol:$CHCl_3$ extraction to remove contaminants, DNA fragments from this area of the gel are ligated into an appropriate plasmid vector and transformed into E. coli. The E. coli clones bearing the mini-library are spread onto agar plates and probed for inserts homologous to FKS1 by in situ colony lysis. DNA from individual transformants is transferred to nitrocellulose, hybridized to a radiolabelled FKS1 DNA fragment, washed, and exposed to film. Colonies containing an insert with homology to FKS1 are visualized on the film; plasmid DNA is then isolated from positive clones, propagated, and analyzed. DNA sequence analysis by standard methods is used to establish the extent of homology to FKS1, and functional homology may be demonstrated by expression in S. cerevisiae disrupted for FKS1.

To isolate FKS2 homologs similar experiments are carried out with an FKS2 probe.

EXAMPLE 24

A. Cloning of Aspergillus homologs of FKS1 and FKS2

Genomic DNA was isolated from A. nidulans FGSCA4, also known as the Glasgow wild-type, and A. nidulans MF5668 by methods known to the art (Tang et al., (1992) Mol. Microbiol., 6:1663-1671). The chromosomal DNA was cut to completion with several restriction enzymes and the digested fragments of DNA were separated by electrophoresis. The fragments of A. fumigatus DNA were transferred to Zeta-Probe GT quaternary amine derivatized nylon membrane which is manufactured by BioRad and the fragments of A. nidulans DNA were transferred to Nytran nylon membrane (S&S; Southern, (1975) J. Mol.Biol., 98:503-517). Duplicate blots of the A. nidulans DNA were prepared. All of the blots were hybridized with ^{32}P probes radiolabeled by random priming (Feinberg and Vogelstein (1983) Anal. Biochem., 132:6-13). The probe for the A. fumigatus blot was a radiolabeled 1.25-kb SalI-ClaI fragment isolated from pJAM54 which contains the FKS1 gene. One A. nidulans blot was also hybridized to this probe and the other A. nidulans blot was hybridized to a radiolabeled 1.7-kb KpnI-PstI fragment from pFF250 which contains a portion of the FKS2 gene. The blots were hybridized overnight under stringent conditions and washed by stringent methods (Maniatis et al., supra). The blots were then exposed to XAR-5 film and developed by conventional methods (Laskey and Mills (1977) FEBS Letters, 82:314-316). Both probes hybridized to fragments of each Aspergillus DNA tested. The blots illustrate that A. nidulans genomic DNA is homologous to both the S. cerevisiae 1.25kb SalI-ClaI fragment from the FKS1 gene and the 1.7-kb KpnI-PstI fragment from the FKS2 gene. A. fumigatus DNA is also homologous to the S. cerevisiae 1.25kb SalI-ClaI fragment from the FKS1 gene.

To clone the A. nidulans homologs, two cosmid libraries of A. nidulans genomic DNA have been obtained from the Fungal Genetics Stock Center. Cosmid vectors are modified plasmids that contain "cos" sequences required for packaging DNA into bacteriophage lambda

particles (Maniatis et al., supra). Cosmids also contain an origin of replication and a drug resistance marker and can be introduced into E. coli by standard transformation procedures and propagated as plasmids. Cos sequences enable 35- to 45-kb fragments of foreign DNA that are ligated to the vector to be packaged into lambda particles and to subsequently circularize upon infection of E. coli. Two complete cosmid libraries were constructed in the vectors LORIST2 and pWE15 by Brody et al., (Nucleic Acids Research, 19:3105-3109). Cosmid pWE15 contains a ColE1 origin of replication whereas LORIST2 contains a bacteriophage lambda origin of replication. DNA sequences that are unstable in one vector are often stable in the other (Evans et al., (1987). Methods in Enzymol., Berger and Kimmel Eds. Academic Press, New York. Vol. 152:604-610). Clones from the cosmid libraries are transferred to Nytran membranes and screened by methods known to the art (Maniatis et al.). The probes are the fragments from the FKS1 and FKS2 genes described above. If FKS1 and FKS2 homologs are absent from the cosmid libraries or the sequences are unstable, additional libraries are screened. If the homologs are absent from preexisting libraries or if only part of the gene is isolated, an A. nidulans genomic Sau3AI partial library is constructed in the Stratagene Vector Lambda Dash using a cloning kit obtained from the manufacturer and methods of the art (Maniatis).

Similar methodology is used to clone the A. fumigatus homologs of FKS1 and FKS2.

B. Isolation of A. nidulans homolog (fksA) of S. cerevisiae FKS1 and FKS2 by cross hybridization

fksA is the designation for an Aspergillus nidulans homolog of FKS1 and FKS2. Homology at the DNA level was demonstrated between the S. cerevisiae FKS1 and FKS2 genes and genomic DNA of A. nidulans. This homology forms the basis of a strategy to clone Aspergillus homologs.

An A. nidulans genomic library constructed in the Stratagene cosmid vector pWE15 (Brody et al., 1991, Nucleic Acids

Research, 19:3105-3109) was obtained from the Fungal Genetics Stock Center. This cosmid library consists of 2,832 individual cosmid containing E. coli transformants divided amongst 30 microtiter plates. One thousand four hundred eighty-eight transformants were transferred to Zeta-Probe GT quaternary amine derivatized nylon membranes (manufactured by BioRad) as colony blots.

The colony blots of the microtiter plates (96 colonies/plate; 1 blot/plate) were made as follows: individual cosmids were grown in LB broth (Maniatis, supra) in microtiter dishes overnight and were subsequently inoculated onto LB agar containing 50 micrograms per ml ampicillin. After seven hours of growth, two colony lifts were made from each plate and the filters were transferred to fresh plates. The colonies were grown an additional four hours and fixed to the filters. The filters were treated with 0.5 N NaOH, neutralized with 1 M Tris pH 7.5/1.5 M NaCl, washed in 1 M Tris pH 7.5/1.5 M NaCl/0.2% SDS, and washed again in 1 M Tris pH 7.5/1.5 M NaCl. Duplicate blots were hybridized with a radiolabeled (^{32}P) 4.0 kb KpnI FKS1 fragment isolated from pJAM54 and a 1.7kb PstI-KpnI fragment isolated from pFF250. All ^{32}P probes were radiolabeled by random priming (Feinberg and Vogelstein (1983) Anal. Biochem. 132:6-13). The blots were hybridized using conditions recommended for Zeta membranes by the manufacturer Biorad. One colony was initially detected with only the FKS2 probe. This cosmid was designated pGS1, and hybridization to the FKS1 and FKS2 genes was subsequently confirmed by DNA slot blot analysis with purified cosmid DNA. Cosmid DNA was isolated from cultures grown for ten hours in LB medium and purified with a Qiagen plasmid maxi kit. Duplicate DNA slot blots were prepared by applying 1.5 micrograms of each sample to a Zeta-Probe GT quaternary amine derivatized nylon membrane (BioRad) with a Minifold II slot blot apparatus according to the directions of the manufacturer (Schleicher and Schuell). The samples were pGS1 DNA, vector pWE15 DNA, and DNA from a nonhybridizing cosmid. The slot blots were hybridized as described for the colony blots. Both the FKS1 and FKS2 probes

hybridized specifically to the pGS1 DNA, and not to DNA from cosmid vector pWE15 or DNA isolated from a nonhybridizing colony.

The insert of cosmid pGS1 was estimated to be ~30kb by restriction endonuclease digestion and agarose gel electrophoresis and was released from the vector by digestion with either NotI or EcoRI. Specific restriction fragments from pGS1 with homology to FKS2 were identified by Southern blot hybridization using the same hybridization conditions. An 11.0 kb EcoRI fragment that hybridized to FKS2 was subcloned into vector Bluescript (Stratagene) to construct subclone pGS3. A restriction map was determined by restriction endonuclease digestion and agarose gel electrophoresis of the restriction fragments and is shown in Figure 3. The region of pGS3 homologous to FKS2 was localized by Southern hybridization of blots of restriction fragments to the FKS2 probe. The 568 bp PstI-EcoRV fragment was determined to be internal to the hybridizing region and specific for fksA based on the following evidence: the 1.7kb PstI fragment bordering on the left and the 2.4kb EcoRV fragment bordering on the right hybridized to FKS2.

As some genomic libraries contain rearranged genes or DNA resulting from ligation of noncontiguous restriction fragments, Southern blot hybridization with the S. cerevisiae FKS2 gene and a homologous probe was performed to determine if cosmid pGS1 and its derivative, pGS3, were colinear with the A. nidulans genome. The homologous probe was the 568 bp fksA specific PstI-EcoRV fragment isolated from pGS2. Plasmid pGS2 was constructed by subcloning a 6.0kb SalI fragment of pGS1 into Bluescript (Stratagene). A. nidulans genomic DNA was digested with SalI, EcoRI, EcoRV, KpnI, and EcoRI/SstII. The hybridization data indicated that the appropriate-sized restriction fragments of genomic DNA were found for enzymes proximal to the EcoRV site of the internal PstI-EcoRV fragment, but restriction fragments of genomic DNA corresponding to restriction sites distal of this EcoRV site were not found. Cosmid pGS1 and its derivative pGS3 are colinear with the A. nidulans genome from the left-

hand EcoRI site to the second EcoRV site of the restriction map of pGS3 shown in Figure 3.

To ensure isolation of a cosmid clone containing the entire A. nidulans fksA gene, another library was screened. An A. nidulans cosmid library constructed in vector pLORIST2 (Brody et al., 1991, Nucleic Acids Research, 19:3105-3109) was obtained from the Fungal Genetics Stock Center. The library was screened exactly as described for the isolation of pGS1, except that the probe was the A. nidulans internal 568bp PstI-EcoRV fragment. One cosmid clone, p11G12, out of 2880 cosmids screened, hybridized strongly with the probe. DNA slot blot analysis with purified cosmid DNA confirmed hybridization to the A. nidulans homologous probe as well as to the FKS2 probe. The homologous probe was the 568 bp PstI-EcoRV fragment isolated from pGS4. Plasmid pGS4 was constructed by subcloning the 568p PstI-EcoRV fragment of pGS3 into Bluescript. Colinearity of p11G12 with A. nidulans genomic DNA was determined by Southern blot hybridization with the FKS2 probe. The restriction enzymes tested were EcoRV-BglII, EcoRV-KpnI, EcoRV-SalI, PstI, SpeI, and XbaI. The restriction fragments obtained with p11G12 corresponded to those obtained with A. nidulans genomic DNA. The data indicated that the 568 bp PstI-EcoRV fragment specific for fksA is flanked on each side by ~7.0kb of DNA that is colinear with the genome. An 11.0 kb XbaI fragment of p11G12 that hybridized to FKS2 and is colinear with the genome was subcloned into Bluescript to construct pGS6. A restriction map was determined by restriction endonuclease digestion and agarose gel electrophoresis of the restriction fragments and is shown in Figure 4.

DNA sequence was determined either manually by the method of Sanger et al., (Proc.Natl Acad. Sci., 74:5463) using a "Sequenase" kit manufactured by United States Biochemical or using the Applied Biosystems Model 373A DNA Sequencing System with a "Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit". Template DNA was obtained from the following plasmids: pGS4, pGS7, pGS6, pGS15, pGS16, pGS17, pGS18, pGS19, pGS20, and pGS21.

Plasmid pGS7 was constructed by subcloning the 11.0 kb XbaI insert of pGS6 into pBR322. The 3.6 kb KpnI fragment and the 2.2 kb XhoI fragment of pGS6 were subcloned into pGEM7 to construct pGS15 and pGS16, respectively. A set of nested deletions was constructed in pGS15 by partial Sau3A digestion using a method described by Gewain et al., (1992, Gene, 119:149). Briefly, plasmid pGS15 was linearized with BamHI which is in the multicloning site of the vector and the DNA was precipitated and resuspended. One microgram aliquots in 15 microliter reaction mixtures were subjected to partial digestion in 1X Sau3A buffer (New England Biolabs). The enzyme was diluted to 0.75 units/microliter in storage buffer (50 mM KCl, 10 mM TrisHCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT, 200 mg/ml BSA, 50% glycerol) and serially diluted two-fold in storage buffer eight more times. One microliter of each dilution as well as the undiluted control was added to each tube containing 14 microliters and the mixture was incubated at 37°C for 30 minutes. Reactions were terminated by the addition of 3.4 microliters of stop buffer containing 50 mM EDTA and heated at 65°C for 20 minutes. The DNA was subjected to electrophoresis and fragments of the appropriate sizes were gel purified with a Qiagen Qiaquick protocol. The fragments were quantitiated and religated with 25 ng of DNA per five microliter ligation reaction. The ligations containing the four largest fragments were precipitated and digested with Csp451 in five microliter reactions. The Csp451 site is between the BamHI and the KpnI site of the vector. This digestion was necessary to eliminate any contaminating fragments that did not contain a deletion. All of the DNA samples were transformed into DH5a F'IQ, and the appropriate recombinants were identified by restriction endonuclease digestion. The deletions contained in plasmids pGS17, pGS18, pGS19, pGS20 and pGS21 are shown in Figure 4.

Sequencing of the 568 bp PstI-EcoRV insert of pGS4 was initiated in both directions using KS and SK sequencing primers which bind to the vector (Stratagene). The fksA sequence was used to design primers to extend the sequence in both directions for each strand of the insert. Primers to each end of the sequence of the 568 bp insert of

pGS4 were made and used to extend the fksA sequence with pGS7 as template. This information was used to design primers to extend the sequence in both directions using DNA from pGS15 and pGS16 as template. Additional 3' sequence was obtained with plasmids pGS17, pGS18, pGS19, pGS20, and pGS21 which contain the nested deletions. Sequencing was initiated using an SP6 primer which binds to the vector (Promega) and in cases where the sequence of the plasmids did not overlap, a primer based on fksA sequence was used to extend the sequence. Primers based on fksA sequence were also used to obtain the sequence of the opposite strand using pGS6 as template. The sequence was assembled and analyzed with the University of Wisconsin Genetics Computer Group (GCG) sequence analysis software package.

The DNA sequence of 2565 nucleotides of fksA was determined with sequence of 1600 nucleotides based on both strands (Figure 5). A putative open reading frame of 855 amino acids was deduced that exhibits 67% identity to S. cerevisiae FKS1 and FKS2 proteins. The amino acid sequences were compared with the GAP™ (GCG) program. The region of FKS2 homologous to fksA extends from amino acid 943 to amino acid 1799, the latter being close to the carboxy terminus. The first half of the fksA putative open reading frame (amino acids 1-427) is most homologous to FKS2 exhibiting 82% identity, whereas the latter half is 53% identical.

Localization of the fksA gene on pGS6 can be determined based on sequence information and transcript mapping. The portion of the fksA gene that has been sequenced begins 311 nucleotides to the left of the fourth PstI site of pGS6 as shown on the restriction map in Figure 4. Based on the homology obtained between the fksA gene product and the Saccharomyces FKS1 and FKS2 gene products, it can be deduced that the direction of transcription of fksA is from left to right on the restriction map of pGS6 shown in Figure 4. The fksA gene was further localized on pGS6 by transcript mapping. Total A. nidulans RNA was isolated by methods known to the art as described by Timberlake (Biol. and Mol. Biol. of Plant-Pathogen Interactions, 1986). The RNA was subjected to electrophoresis in 1.5% agarose, 2.2 M

formaldehyde, 1X MOPS buffer, transferred to nytran nylon membranes (Schleicher and Schuell) and hybridized according to a protocol of Gelman Sciences (Protocol Number 6, Application Protocols for BioTrace Binding Matrices). Hybridization of the fksA specific 568 bp PstI-EcoRV fragment of pGS4 to the gel blot detected a single transcript. An identical-sized transcript was detected by the two proximal PstI fragments of pGS6, a 1.2kb PstI fragment and a 0.7kb PstI fragment, but no transcript was detected with the 1.4 kb PstI-SpeI fragment of pGS3 which is 5' to the 1.2kb PstI-PstI fragment (the 1.4 kb PstI-SpeI fragment was isolated from pGS9 which was constructed by subcloning the fragment from pGS3 into Bluescript). These data indicate that the fksA transcript begins within the 1.2kb PstI-PstI fragment. The sequence data indicates that the fksA gene extends beyond the EcoRV site of pGS6. The 1.6 kb NdeI-NheI fragment of pGS6 did not detect a transcript, indicating that the transcript ends before the NdeI site. To summarize, the fksA transcript begins within the 1.2kb PstI fragment and ends between the EcoRV and second NdeI site of pGS6. It is possible that regulatory sequences of the fksA gene are located 5' of the 1.2 kb PstI fragment.

EXAMPLE 25

Isolation of FKS homologs from phytopathogenic fungi

To clone FKS1 and FKS2 homologs from phytopathogenic fungi such as *Magnaporthe grisea* and *Ustilago maydis*, high molecular weight genomic DNA is isolated by the method described by Atkins and Lambowitz (Mol. Cell. Biol., 5:2272-2278), partially digested by the restriction enzyme, Sau3AI, and cloned into the Stratagene Vector Lambda-Dash using a cloning kit obtained from the manufacturer and methods of the art (Maniatis). The libraries are screened using probes from FKS1 and FKS2 essentially as described above.

EXAMPLE 26

A. Isolation of the pcr1 (fks2-1) mutant

The L-733,560 resistant mutant MY2256 (also known as YFK0978 and YM0148) was isolated from strain YFK0931-07B using standard procedures. Four congenic (fks1-1) parental strains (YFK0931-03B, YFK0931-07B, YFK0931-10C, and YFK0932-01C) were used in the mutant hunt. The genotypes of the four strains are listed below. The strains contain plasmid pDL1 which contains an ARS element, a centromere and the CNB1, SUP11, and URA3 genes.

This mutant hunt was designed to identify mutations in the FKS2 gene that confer echinocandin resistance. Briefly, the parental strains were grown overnight in 5 ml of YPAD10Ca medium (YPAD medium containing 10 mM $CaCl_2$) at 28°C. Cells were diluted to 1 x 10^3 cells/ml in YPAD10Ca, and aliquots (0.2 ml) of the cultures were dispensed into 96 individual microtiter wells. The cultures were grown to saturation at 28°C. Cells from five wells were diluted 1:20 and the optical density at 660 nm (OD_{660}) was determined to calculate the average cell density for each culture (1 OD_{660} = 3.3 x 10^7 cells/ml).

Forty cultures of each strain were plated on YPAD10Ca medium containing 1 mcg/ml L-733,560. In addition 20 wells of YFK932-1C and YFK931-10C were diluted 1:10 and 1:100 and plated on YPAD10Ca medium containing 1mcg/ml L-733,560. The plates were incubated at 28°C. Two colonies were picked from each drug plate, clonally purified on -Ura and YPAG medium and grown at 28°C. Two independent clones from each plate were picked to master plates of -Ura and YPAD10Ca media. The master plates were replica plated to standard drop-out medium, YPAD10Ca medium, and to YPAD10Ca medium containing either 1 mcg/ml of FK520, FK506, L-733,560, 10 mg/ml Cyclosporin A, or 0.1 mcg/ml rapamycin. The plates were incubated at 28°C. Temperature sensitivity was determined by replica plating the masters to YPAD medium and incubating the plates at 37°C. The plates were scored after two and three days.

From this experiment, eighteen independent mutants that grew on YPAD10Ca medium containing L-733,560 (1 mcg/ml) were identified from approximately 3.7 x 10^9 cells screened. These pcr (pneumocandin resistant) mutants were resistant to L-733,560 and sensitive to the immunosuppressants FK506, FK520, CsA, and rapamycin. One of the mutants (MY2256) also possessed a temperature sensitive phenotype at 37°C. The sensitivities of MY2256 and its parent strain (YFK0931-07B) to L-733,560, FK520, FK506, CsA, and rapamycin were measured, and the results are shown below. As shown in the table below, the mutant is significantly more resistant to L-733,560 than its parent. MY2256 and YFK0931-07B exhibit similar sensitivities to the immunosuppressants tested.

Mixed membrane fractions were prepared from MY2256 and YFK0931-07B and the sensitivity of 1,3-beta-D-glucan synthase activity to L-733,560 was assayed in the partially purified membrane preparations using standard procedures. The specific activities of 1,3-beta-D-glucan synthase activity from YFK0931-07B and MY2256 were 60 and 45 nmoles of UDP-D-[6-^{3}H]Glucose incorporated mg^{-1} hr^{-1}. The IC_{50} of the enzyme activity from the mutant and parental strains were 16-24 mcM and 0.21 mcM, respectively, indicating that 1,3-beta-D-glucan synthase activity in MY2256 is resistant to L-733,560. MY2256 was further characterized.

EXAMPLE 27

Genetic characterization of the pcr1 mutant

MY2256 (MATa fks1-1 pcr1) was crossed to the wild type strain YFK0005 (MATalpha FKS1+ PCR1+) to generate strain YFK0996-11B. YFK0996-11B (MATa fks1-1 pcr1) was mated to YFK0688-14B (MATalpha fks1-1 PCR1+), sporulated and dissected. In the 29 four-spore and 12 three-spore tetrads from this cross, the pcr1 phenotype (resistance to L-733,560) segregated 2^r:2^s indicating that the pcr1 phenotype is the result of a single mutation. Strains MY2259 (also known as YFK1087-20B, MATalpha fks1-1 pcr1) and MY2260 (also

known as YFK1087-20A, MATa fks1-1 pcr1) were generated from this cross. Like the original MY2256 mutant, MY2259 and MY2260 contain the fks1-1 and pcr1 mutations. However, these strains do not contain plasmid pDL1 that was present in the original mutant.

YFK0996-11B (MATa fks1-1 pcr1) was also crossed to YFK0005 (MATalpha FKS1 PCR1). In this cross, the pcr1 and fks1-1 mutations segregate independently. In the 16 four-spore and 20 three-spore tetrads, the segregation pattern of 1 Parental Ditype : 1 Non Parental Ditype : 4 Tetratype tetrads is indicative of two unlinked genes. This cross also demonstrated that the pcr1 phenotype is expressed in an FKS1 background. FKS1 pcr1 spores are resistant to L-733,560 and the calcineurin inhibitors FK520, FK506, and CsA. Strains MY2257 (also known as YFK1088-23B, MATa FKS1+ pcr1), MY2258 (also known as YFK1088-16D, MATalpha FKS1+ pcr1), and YFK1088-02D (MATa FKS1+ pcr1) were segregants from this cross. As shown in the table below, these segregants contain the pcr1 mutation in a wild-type FKS1 background and lack plasmid pDL1 present in the original mutant.

To determine if the pcr1 mutation mapped to the FKS2 gene, YFK1088-02D (MATa pcr1) was crossed to YFF2720 (MATalpha fks2::TRP1). In the 31 four-spore and 6 three-spore tetrads from this cross, all segregants demonstrated the parental phenotypes of resistance to L-733,560 (pcr1) and tryptophan auxotrophy (trp1) or sensitivity to L-733,560 and tryptophan prototrophy (fks2::TRP1). These results demonstrate that the pcr1 mutation is tightly linked to the FKS2 gene. In two additional crosses, YFK0996-23D (MATa pcr1 cnb1::LYS2) was mated to YFF2720 (MATalpha fks2::TRP1) and to YFF2721 (MATalpha fks2::TRP1). In the 78 tetrads tested, all of the fks2::TRP1 spores were sensitive to L-733,560 supporting the model that the pcr1 mutation maps to the FKS2 gene. Moreover, all of the cnb1::LYS2 spores from these crosses were sensitive to L-733,560. This would be expected if the mutation maps within the calcineurin-regulated FKS2 gene.

In summary, the pcr1 mutation is a single gene, segregates independently of fks1-1, is expressed in an FKS1 cell, and is tightly linked to the FKS2 gene. Accordingly, the pcr1 allele has been renamed as fks2-1.

B. Quantitating the level and spectrum of drug resistance of the pcr1(fks2-1) mutant

The sensitivities of pcr1(fks2-1) fks1-1 and pcr1(fks2-1) FKS1 strains to L-733,560, L-636,947 (Aculeacin), and L-687,781 (Dihydropapulocandin) were determined in MIC assays. Briefly, strains were grown to stationary phase in 5.0 ml of liquid YPAD medium. MY2256 precultures were grown in liquid YPAD10Ca. MIC assays were performed in flat well microtiter plates in triplicate. Each well of the microtiter plate was filled with 100 mcL of YPAD medium. To the first well, 100 mcL of a 4X solution of drug in YPAD medium was added. To serve as a control, a stock solution of 160 mcL DMSO per mcL of YPAD was made. 100 mcL of this solution was added to the initial well for strains grown in the presence of solvent but the absence of drug. Two-fold serial dilutions of the drug were performed down the plate.

Cultures were diluted to 5×10^5 cells/ml in YPAD (1 $OD_{660} = 3.3 \times 10^7$ cells/ml). 100 mcL of diluted culture was added to each well, resuspended, and incubated at 28°C. After 42 hours, cultures were resuspended and cells densities measured in an SLT Laboratories 340 ATTC microtiter plate reader. The MICs concentrations presented in the table below represent the concentrations of drug that result in less than 10% growth of the strain grown in the absence of drug.

TABLE

	MIC (ng/ml)				
Strain	L-733,560	FK506	FK520	CsA	Rapamycin
YFK0931-07B	30	30	60	5000	7
MY2256	4000	60	60	5000	7

TABLE

	Tetrad Analysis of *pcr1 vs fks1-1*			
Genotype/Strains	Parental Ditype	Nonparental Ditype	Tetratype	
pcr1 fks1-1 x FKS1 YFK0996-11B	5	1	10	*four-spored*
x YFK0005	1	5	14	*three-spored*
	6	6	24	*total*
	(6)	(6)	(24)	*expected*

TABLE

	MIC (mcg/ml)		
Genotype / Strain	L-733,560	L-636,947 (Aculeacin)	L-687,781 (Dihydropapulocandin)
Wild type YFK0005	0.1	1	40
fks1-1 PCR1 YFK0688-14B	0.05	0.5	10
fks1-1 pcr1(fks2-1) MY2256 YFK0996-11B MY2259 MY2260	4	>40	>40
FKS1 pcr1(fks2-1) MY2257 MY2258	0.625	>40	>40

EXAMPLE 28

Cloning and Expression of 1,3-beta-D-glucan synthase subunit cDNA into Bacterial Expression Vectors

Recombinant 1,3-beta-D-glucan synthase subunit is produced in a bacterial expression system such as E. coli. The 1,3-beta-D-glucan synthase subunit expression cassette is transferred into an E. coli expression vector; expression vectors include but are not limited to, the pET series (Novagen). The pET vectors place 1,3-beta-D-glucan synthase subunit expression under control of the tightly regulated bacteriophage T7 promoter. Following transfer of this construct into an E. coli host which contains a chromosomal copy of the T7 RNA polymerase gene driven by the inducible lac promoter, expression of 1,3-beta-D-glucan synthase subunit is induced by addition of an appropriate lac substrate (IPTG) is added to the culture. The levels of expressed 1,3-beta-D-glucan synthase subunit are determined by the assays described herein.

EXAMPLE 29

Cloning and Expression of 1,3-beta-D-glucan synthase subunit cDNA into a Vector for Expression in Insect Cells

Baculovirus vectors derived from the genome of the AcNPV virus are designed to provide high level expression of cDNA in the Sf9 line of insect cells (ATCC CRL# 1711). Recombinant baculovirus expressing 1,3-beta-D-glucan synthase subunit cDNA is produced by the following standard methods (InVitrogen Maxbac Manual): the 1,3-beta-D-glucan synthase subunit cDNA constructs are ligated into the polyhedrin gene in a variety of baculovirus transfer vectors, including the pAC360 and the BlueBac vector (InVitrogen). Recombinant baculoviruses are generated by homologous recombination following co-transfection of the baculovirus transfer vector and linearized AcNPV genomic DNA (Kitts, P.A., Nuc. Acid. Res., 18, 5667 (1990)) into Sf9 cells. Recombinant pAC360 viruses are identified

by the absence of inclusion bodies in infected cells and recombinant pBlueBac viruses are identified on the basis of β-galactosidase expression (Summers, M.D. and Smith, G.E., Texas Agriculture Exp. Station Bulletin No. 1555). Following plaque purification, 1,3-beta-D-glucan synthase subunit expression is measured.

Authentic 1,3-beta-D-glucan synthase subunit receptor is found in association with the infected cells. Active 1,3-beta-D-glucan synthase subunit is extracted from infected cells by hypotonic or detergent lysis.

Alternatively, the 1,3-beta-D-glucan synthase subunit is expressed in the Drosophila Schneider 2 cell line by cotransfection of the Schneider 2 cells with a vector containing the modified receptor DNA downstream and under control of an inducible metallothionin promoter, and a vector encoding the G418 resistant neomycin gene. Following growth in the presence of G418, resistant cells are obtained and induced to express 1,3-beta-D-glucan synthase subunit by the addition of $CuSO_4$. Identification of modulators of the 1,3-beta-D-glucan synthase subunit is accomplished by assays using either whole cells or membrane preparations.

EXAMPLE 30

Purification of Recombinant 1,3-beta-D-glucan synthase subunit

Recombinantly produced 1,3-beta-D-glucan synthase subunit may be purified by a variety of procedures, including but not limited to antibody affinity chromatography.

Recombinant 1,3-beta-D-glucan synthase subunit antibody affinity columns are made by adding the anti-1,3-beta-D-glucan synthase subunit antibodies to Affigel-10 (Biorad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1 M ethanolamine HCl (pH 8). The column is washed with water followed

by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) together with appropriate membrane solubilizing agents such as detergents, and the cell culture supernatants or cell extracts containing solubilized 1,3-beta-D-glucan synthase subunit is slowly passed through the column. The column is then washed with phosphate-buffered saline (PBS) supplemented with detergents until the optical density (A_{280}) falls to background; then the protein is eluted with 0.23 M glycine-HCl (pH 2.6) supplemented with detergents. The purified 1,3-beta-D-glucan synthase subunit protein is then dialyzed against PBS.

EXAMPLE 31

Cloning and Expression of 1,3-beta-D-glucan synthase subunit in Mammalian Cell System

1,3-beta-D-glucan synthase subunit is cloned into a mammalian expression vector. The mammalian expression vector is used to transform a mammalian cell line to produce a recombinant mammalian cell line. The recombinant mammalian cell line is cultivated under conditions that permit expression of the 1,3-beta-D-glucan synthase subunit. The recombinant mammalian cell line or membranes isolated from the recombinant mammalian cell line are used in assays to identify compounds that bind to the recombinant 1,3-beta-D-glucan synthase subunit.

EXAMPLE 32

Screening Assay

Recombinant cells containing DNA encoding a 1,3-beta-D-glucan synthase subunit, membranes derived from the recombinant cells, or recombinant 1,3-beta-D-glucan synthase subunit preparations derived from the cells or membranes may be used to identify compounds that modulate 1,3-beta-D-glucan synthase subunit activity.

Modulation of such activity may occur at the level of DNA, RNA, protein or combinations thereof. One method of identifying compounds that modulate 1,3-beta-D-glucan synthase subunit comprises:

(a) mixing a test compound with a solution containing 1,3-beta-D-glucan synthase subunit to form a mixture;
(b) measuring 1,3-beta-D-glucan synthase subunit activity in the mixture; and
(c) comparing the 1,3-beta-D-glucan synthase subunit activity of the mixture to a standard.

EXAMPLE 33

DNA Sequence of FKS1

The DNA sequence of FKS1 was determined and is shown in Figure 6.

EXAMPLE 34

Amino Acid Sequence of FKS1

The amino acid sequence of FKS1 was determined and is shown in Figure 7.

EXAMPLE 35

DNA Sequence of FKS2

The DNA sequence of FKS2 was determined and is shown in Figure 8.

EXAMPLE 36

Amino Acid Sequence of FKS2

The amino acid sequence of FKS2 was determined and is shown in Figure 9.

EXAMPLE 37

To identify the fks1-1 mutation in strain R560-1C, gapped plasmids lacking a portion of the FKS1 coding sequence were prepared from plasmid pJAM54 by digestion with restriction enzymes (including but not limited to KpnI, SstI, BglII, XhoI) and purification by agarose gel electrophoresis. The gapped plasmids were purified from the gel using standard methods and transformed into strain R560-1C. Sixty Ura+ transformants selected on uracil dropout medium were patched onto the same medium, grown for 24 h at 30°C, then replica plated to uracil dropout medium supplemented with 4 μg/ml L-733,560 and incubated for 2 days at 30°C. Growth of the clones on uracil-free drug-containing plates would suggest that: 1) the gapped plasmid was repaired at the ends of the gap through homologous recombination with the chromosome of strain R560-1C; and 2) the gap spanned the fks1-2 mutation. In contrast, if the gap spanned a region of the chromosome which did not contain the fks1-2 mutation, the repaired plasmid would carry the intact wild-type FKS1 gene, and the transformants would be partially drug-sensitive. Fifty-six of the sixty clones transformed with the KpnI-gapped version of pJAM54 were drug resistant. Plasmid DNA from these clones was isolated, amplified by propagation in E. coli, and transformed into YLIP137, a yeast strain with an insertion - deletion in the chromosomal copy of FKS1. Strain YLIP137 is phenotypically similar to strain YFF2409 described in Example 1, i.e., the chromosomal copy of FKS1 in YLIP137 has been functionally inactivated. The plasmid-borne copy of FKS1 is the only functional copy of FKS1 in these cells; if they are resistant to L-733,560, it must be because the plasmid carries the fks1-2 mutant version of FKS1. Ura+ transformants of YLIP137 were selected on uracil dropout medium, and several clones were analyzed for susceptibility to L-733,560 by liquid MIC assays. All clones were as resistant to the drug as the original R560-1C mutant. We have designated the original gap-repaired plasmid carrying the fks1-2 mutation pJAM67.

The KpnI restriction fragment from plasmid pJAM67 is 3.5 kb in length. To identify a smaller fragment bearing the fks1-2 mutation, the fragments of the FKS1gene in plasmid pJAM54 were replaced with the corresponding fragment from pJAM67 (fks1-2), transformed the new constructs into YLIP137, and assayed the clones for drug resistance using liquid MIC assays. In this manner, it was determined that the fks1-2 mutation was within a ca. 0.8-kb SalI-NcoI fragment of pJAM67. This fragment was subcloned into an E. coli plasmid suitable for DNA sequencing (pGEM3(z)f).

The sequence of the ca. 0.8-kb SalI-NcoI fragment was determined using the Model XXXX Automated DNA sequencer from Applied Biosystems, Inc, as per the manufacturer's specifications. Sequence data was analyzed using the GCG software package from the Genetics Computing Group, Madison Wisconsin. Comparison of the DNA sequence of the SalI-NcoI fragment (exact length = 711 bp) from pJAM67 to that of FKS1 revealed a single change. At nucleotide position 469 of the FKS1 SalI-NcoI fragment, the base is T (thymine); in the fks1-2 DNA fragment, the nucleotide base at the corresponding position is A (adenine). When translated into protein, this change results in the substitution of isoleucine (Fks1-2p) for phenylalanine (Fks1p) at position 639 of the 1877 amino acid protein primary sequence. One hypothesis is that this change is responsible for the L-733,560 resistance of both strain R560-1C and the 1,3-b-D-glucan synthase activity derived from it.

EXAMPLE 38

Total genomic DNA from Candida albicans ATCC10261 was digested to completion with BamHI and KpnI and separated by agarose gel electrophoresis using a 0.8% gel. A portion of the DNA fragments from the gel was transfered to a nitrocellulose filter and probed with a 1.25-kbSalI- ClaI fragment from S. cerevisiae FKS1 by Southern blotting (Maniatis, supra). A ca. 2-kb fragment of Candida DNA hybridized to the probe, and the fragments from the

corresponding region of the remainder of the gel were excised, purified by standard methods, and ligated into vector pGEM3(z)f (Stratagene) digested with BamHI and KpnI. The ligation mixture was transformed into competent cells of E. coli, and plasmid-bearing transformants were selected on medium containing ampicillin and pooled. To identify clones which carried a plasmid with the 2-kb C. albicans FKS1-homologous DNA, aliquots of the pooled transformants were spread on selective medium, and colonies were transferred to nitrocellulose, lysed by standard methods, and probed with the [^{32}P]-labeled 1.25-kb SalI-ClaI fragment isolated from pJAM54. Filters were washed under stringent conditions and exposed to film. Nineteen colonies appeared to give a positive signal on the blot. Using the original colony as a source, cells from each of the potential clones were grown in liquid medium and plasmid DNA was isolated. The DNA was digested with KpnI and BamHI, and fragments separated on 0.8% agarose gels were probed with the radiolabeled 1.25-kb SalI- ClaI fragment from pJAM54 by Southern blotting. Three of the nineteen plasmids contained a ca. 2-kb fragment which hybridized intensely with the probe. The plasmid with the KpnI-BamHI fragment of the C. albicans FKS1 homolog has been designated as pGJS1. The Candida gene gene which is homologous to FKS1 was designated FKS1can.

The nucleotide sequence of the ca. 2-kb fragment from pGJS1 was determined using standard methods. For the first two sequencing reactions, denatured pGJS1 DNA was annealed to the "T7 primer" and "SP6 primer" available from Stratagene. All other reagents, including the enzyme "Sequenase v. 2", were from U.S. Biochemicals and were used according to the manufacturer's specifications. The DNA sequence results from the first reactions were used to design 18-base oligonucleotide primers which were complementary to the "end" of the sequence from these first reactions. These primers were used in the next set of reactions. The process was continued until a contiguous protein-encoding open reading frame could be generated from the data from individual sequencing reactions, using

the GCG analysis programs from the Genetics Computing Group (Madison, Wisconsin).

The predicted peptide sequence of the C. albicans FKS homolog (Fksc1p) was compared to the protein sequence of Fks1p from S. cerevisiae. Amino acids 1 through 689 of Fksc1p aligned with residues 460 through 1147 of Fks1p, using the GAP program of the Genetics Computing Group. The two peptide sequences were 79% identical and 88% similar to one another over this range. This constitutes a very high degree of homology and suggests that the two proteins are very likely to be functionally similar. In particular, phenylalanine at position 639 of Fks1p, which was identified in the mutant gene fks1-2 as a residue important for wild-type susceptibility to echinocandin inhibition (supra) was identical to phenylalanine 180 of the Fksc1p amino acid sequence given in Figure CD1. It is believed that: 1) FKS1can encodes an echinocandin-sensitive subunit of the C. albicans 1,3-beta-D-glucan synthase; 2) the remainder of the Fksc1p protein sequence will show a similar degree of homology to Fks1p; 3) Mutations in FKS1can similar but not limited to the fks1-2 mutation will result in decreased susceptibility of both enzyme activity (1,3-beta-D-glucan synthase containing the mutant Fksc1p subunit) and whole cells (C. albicans cells expressing the mutant Fksc1p) to echinocandin inhibition.

EXAMPLE 39

The effect of loss of a functional copy of either FKS1 or FKS2 on sensitivity to yeast killer toxin was evaluated. The toxin-susceptibility test requires that the test strain lack the M_1 killer virus, since strains containing the virus produce toxin (K^+) and are immune (I^+) to its action, and it is not possible to distinguish the killer resistant (Kre^-) phenotype from the immune (I^+) phenotype. The strains constructed with insertion - deletions of either FKS1(YLIP179 and YLIP183; fks1::HIS3) or FKS2 (YLIP186 and YLIP190; fks2::TRP1) were K^+I^+; therefore, the M_1 virus had to be cured from the strains

before the Kre phenotype could be assayed. YLIP179, YLIP183, YLIP186 and YLIP190 were grown overnight at 37°C. The next day, an aliquot of the overnight culture was transferred to fresh medium (1:1000 dilution) and incubation at 37°C was continued. After three passages, cells from the culture were streaked onto agar plates, and single colonies were isolated and tested for failure to produce killer toxin in a patch assay. The patch assay was performed by: 1) Adding 1 x 10^5 logarithmic-phase cells of the killer toxin supersensitive strain S6 to molten YPAD agar containing 0.25 M citrate buffer, pH 4.7 and 0.03% methylene blue (YPAD Cit MB); 2) Pouring plates and allowing the seeded agar to solidify; 3) Applying a patch of the test strain to the surface of the plate; and 4) Incubating at 25°C for 24 h and looking for a zone of clearing around the patch. Strains which failed to produce a zone were not expressing active toxin (K^-) and were not immune (I^-). Derivatives of YLIP179, YLIP183, YLIP186, and YLIP190 cured of the M1-killer virus by this procedure were tested for susceptibility to killer toxin by a modification of the patch assay. Each test strain was seeded in molten YPAD Cit MB agar and a superkiller strain (K12) was applied as the patch. Under these conditions, there is little to no zone in the lawn of cells when the test strain is Kre^-. All of the K^- I^- isolates derived from fks1::HIS3 strains and fks2::TRP1 strains were sensitive to the toxin produced by strain K12; control assays with several known Kre^- strains (S706, S708, and S726; described in U.S. Patent 5,194,600, Tables I and VI) performed under the same conditions showed little to no zone. Therefore, loss-of-functions mutations in etiher FKS1 or FKS2 resulted in cells which were phenotypically distinct from strains with loss of function mutations in any of the KRE genes described in U.S. patent No. 5,194,600.

EXAMPLE 40

The susceptibility of two different kre mutants to inhibitors of 1,3-b-D-glucan synthase was measured. Strains S442(KRE1 KRE5) S708 (kre1-3) and S726 (kre5-1) were grown in liquid YPAD medium

to stationary phase then seeded in molten YPAD agar at a final concentration of 1 x 10^5 cells per ml before pouring into petri plates. To test for drug sensitivity, pneumocandin B_0, echinocandin B, dihydropapulacandin, and L-733,560 (four known inhibitors of 1,3-beta-D-glucan synthase) were applied to the surface of the plates, and the diameter of each zone of growth inhibition was measured after growth at 30°C for 24 h. The methodology for this assay is essentially as described above, and strains R560-1C, W303-1A, YLIP179 (fks1::HIS3) and YLIP186 (fks2::TRP1) were tested under the same conditions for comparison. Zone diameter is usually a good indicator of susceptibility to an inhibitor and can be used to score resistance or hypersensitivity relative to a congenic wild-type strain. Using these criteria, R560-1C cells were resistant, YLIP179 cells were hypersensitive, and YLIP186 cells were like the wild-type strain in susceptibility to the four 1,3-beta-D-glucan synthase inhibitors. In contrast, the kre mutants [S708 (kre1-3) and S726 (kre5-1)] were equivalent to their wild-type parent strain (S442) in susceptibility to all four compounds. Therefore, there was no affect of the kre mutations on sensitivity to these 1,3-beta-D-glucan synthase inhibitors, while mutant alleles of FKS1 resulted in either resistance (fks1-2) or hypersensitivity (fks1::HIS3) to these compounds. The results imply that a microbial assay for inhibitors of 1,3-beta-D-glucan synthase based on differential susceptibility of a mutant/wild-type strain pair would not be effective with these kre mutants but could be effective with these fks1 mutants.

WHAT IS CLAIMED IS:

1. An essentially pure DNA molecule selected from the group FKS1 and functional derivatives thereof and FKS2 and functional derivatives thereof.

2. The DNA molecule of Claim 1 which is isolated from a microorganism.

3. The DNA molecule of Claim 2, wherein the microorganism is selected from the group consisting of Aspergillus fumigatus, Aspergillus nidulans, Candida albicans, Cryptococcus neoformans, Pneumocystis carinii and Saccharomyces cerevisiae.

4. The DNA molecule of Claim 2, wherein the microorganism is Saccharomyces cerevisiae.

5. The DNA molecule of Claim 1 having a nucleic acid sequence selected from the sequence of Figure 6, the sequence of Figure 8, the DNA sequence of Figure 10, the sequence of Figure 11, and functional derivatives thereof.

6. The DNA molecule of Claim 1 having the restriction map selected from Figure 1, Figure 2, Figure 3 and Figure 4.

7. The DNA molecule of Claim 1, wherein the DNA molecule is operably linked to regulatory sequences such that the DNA may be expressed upon introduction into a prokaryotic or eukaryotic cell.

8. An essentially purified protein encoded by the DNA molecule of Claim 1.

9. The protein of Claim 8 having an amino acid sequence selected from the amino acid sequence of Figure 5, the sequence of Figure 7, the sequence of Figure 9, the amino acid sequence of Figure 10, the sequence of Figure 12 and functional derivatives thereof.

10. A cell containing the DNA molecule of Claim 1.

11. A microorganism selected from the group consisting of YFK532-7C, R560-1C, MS14, YFK0978, YFK1088-23B, YFK1088-16D, YFK1087-20B, and YFK1087-20A.

12. Antibodies to the purified protein of Claim 8.

13. A method for identifying compounds that modulate glucan synthase activity comprising:

(a) cultivating two microorganisms, the first microorganism bearing intact FKS1 DNA and FKS2 DNA, and the second microorganism bearing an altered FKS1 DNA or an altered FKS2 DNA;
(b) incubating aliquots of the microorganisms of step (a) with a quantifiable amount of a compound know to affect glucan synthase;
(c) incubating aliquots of the microorganisms of step (a) with test compounds; and
(d) measuring the glucan synthase activity in the microorganisms of step (b) and step (c).

14. Compounds identified by the method of Claim 13.

15. A method of identifying compounds that affect calcineurin comprising:

(a) cultivating three microorganisms, the first microorganism bearing intact FKS1 DNA and FKS2

(a) cultivating three microorganisms, the first microorganism bearing intact FKS1 DNA and FKS2 DNA, the second microorganism bearing an altered form of FKS1 DNA, and the third microorganism bearing an altered form of FKS1 DNA and at least two copies of CNA2 DNA and at least two copies of CNB2 DNA;

(b) incubating aliquots of the three microorganisms of step (a) with a quantifiable amount of a compound known to affect calcineurin;

(c) incubating aliquots of the three microorganisms with test compounds; and

(d) measuring the relative growth of the aliquots of step (b) and step (c).

16. Compounds identified by the method of Claim 15.

17. Pharmaceutical compositions comprising the compounds of Claim 16.

18. A method of identifying compounds that modulate 1,3-beta-D glucan synthase subunit activity, comprising:

(a) mixing a test compound with a solution containing 1,3-beta-D glucan synthase subunit to form a mixture;

(b) measuring 1,3-beta-D glucan synthase subunit activity in the mixture; and

(c) comparing the 1,3-beta-D glucan synthase subunit activity of the mixture to a standard.

19. Compounds identified by the method of Claim 18.

20. Pharmaceutical compositions comprising the compounds of Claim 19.

21. A method of treating a subject in need of such treatment which comprises administration of the compositions of Claim 20 to the subject.

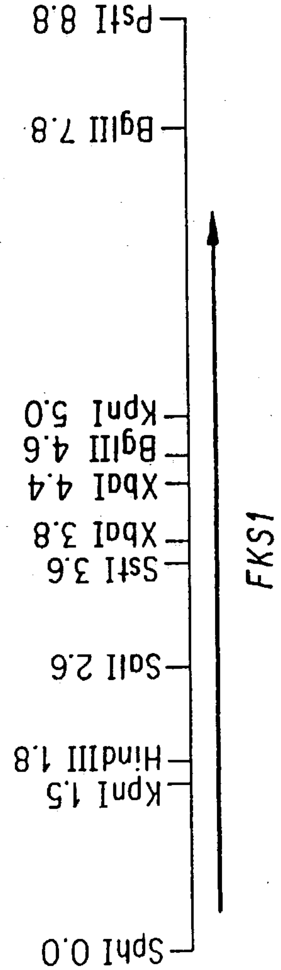
SphI 0.0
KpnI 1.5
HindIII 1.8
SalI 2.6
SstI 3.6
XbaI 3.8
XbaI 4.4
BglII 4.6
KpnI 5.0
BglII 7.8
PstI 8.8
FKS1

FIG. 1

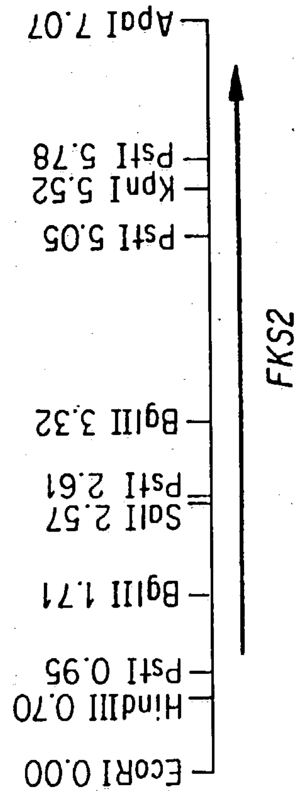
EcoRI 0.00
HindIII 0.70
PstI 0.95
BglII 1.71
SalI 2.57
PstI 2.61
BglII 3.32
PstI 5.05
KpnI 5.52
PstI 5.78
ApaI 7.07
FKS2

FIG. 2

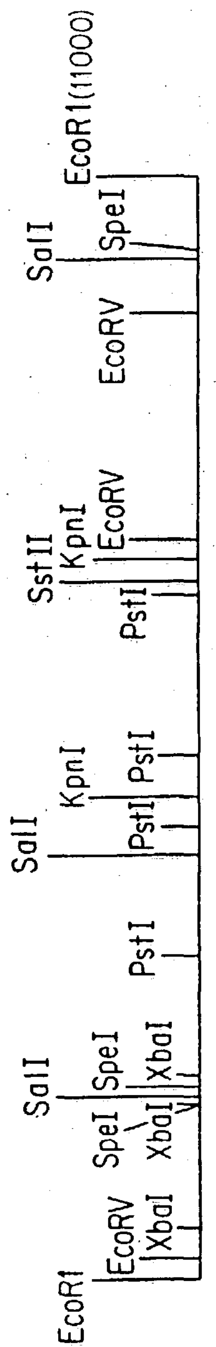
EcoR1
EcoRV
XbaI
SpeI
XbaI
SalI
SpeI
XbaI
PstI
SalI
PstI
KpnI
PstI
PstI
SstII
KpnI
EcoRV
EcoRV
SalI
SpeI
EcoR1(11000)

FIG. 3

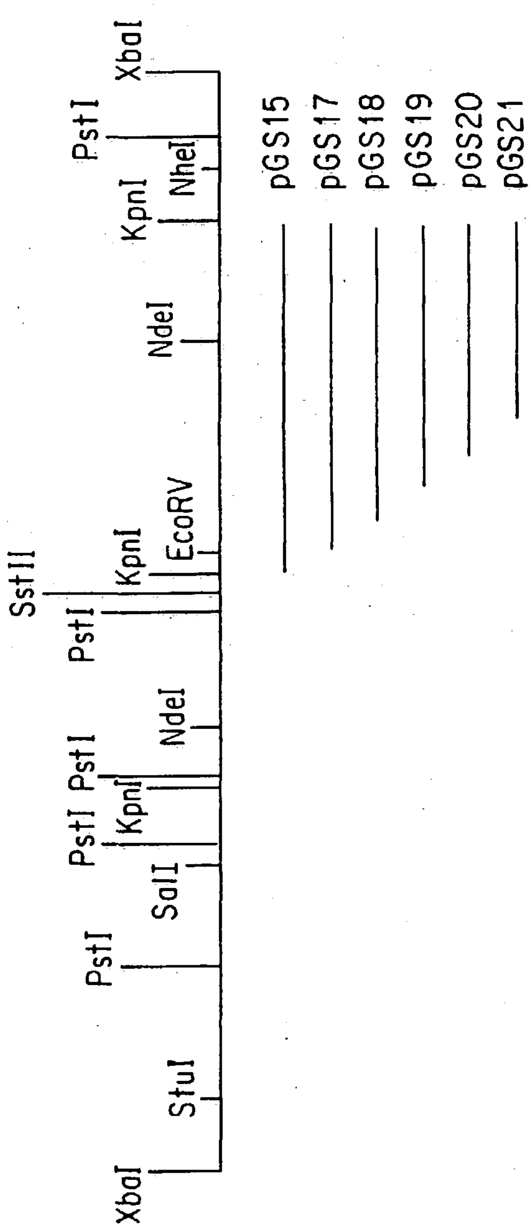
XbaI
StuI
PstI
SalI
PstI
KpnI
PstI
NdeI
PstI
SstII
KpnI
EcoRV
NdeI
KpnI
NheI
PstI
XbaI
pGS15
pGS17
pGS18
pGS19
pGS20
pGS21

FIG. 4

```
  1 TACTGTATCGGTTTCAAGTCTGCTGCTCCCGAGTACACGCTTCGCACCCGTATTTGGTCC  60
  1  Y  C  I  G  F  K  S  A  A  P  E  Y  T  L  R  T  R  I  W  S  20

 61 TCGCTGCGTTCGCAAACTCTTTACAGAACTGTATCCGGGATGATGAACTATAGCAGAGCT 120
 21  S  L  R  S  Q  T  L  Y  R  T  V  S  G  M  M  N  Y  S  R  A  40

121 ATCAAGCTCCTCTACCGTGTGGAGAACCCGGAAGTCGTCCAGATGTTCGGTGGTAATTCT 180
 41  I  K  L  L  Y  R  V  E  N  P  E  V  V  Q  M  F  G  G  N  S  60

181 GAGAAGCTGGAACATGAGCTCGAGAGGATGGCCCGTCGCAAGTTCAAGATCTGTGTTTCA 240
 61  E  K  L  E  H  E  L  E  R  M  A  R  R  K  F  K  I  C  V  S  80

241 ATGCAGCGGTATGCCAAATTCACAAAAGAAGAACGTGAGAACACAGAGTTCCTCCTCCGA 300
 81  M  Q  R  Y  A  K  F  T  K  E  E  R  E  N  T  E  F  L  L  R 100

301 GCCTACCCCGACCTGCAGATTGCCTATCTCGATGAGGAACCTCCAGCCAACGAGGGTGAA 360
101  A  Y  P  D  L  Q  I  A  Y  L  D  E  E  P  P  A  N  E  G  E 120

361 GAGCCGCGTCTCTACTCTGCTTTGATTGATGGACACTGTGAGCTGCTCGAGAATGGCATG 420
121  E  P  R  L  Y  S  A  L  I  D  G  H  C  E  L  L  E  N  G  M 140

421 CGGAAGCCCAAGTTCAGGATCCAGCTCTCCGGAAACCCGATCCTTGGTGACGGCAAGTCT 480
141  R  K  P  K  F  R  I  Q  L  S  G  N  P  I  L  G  D  G  K  S 160

481 GACAACCAAAACCACTCGATCATTTTCTACCGCGGTGAATACATTCAGGTCATTGATGCC 540
161  D  N  Q  N  H  S  I  I  F  Y  R  G  E  Y  I  Q  V  I  D  A 180

541 AACCAAGACAACTATCTCGAAGAGTGCTTGAAAATCCGAAGCGTTCTTGCTGAGTTTGAG 600
181  N  Q  D  N  Y  L  E  E  C  L  K  I  R  S  V  L  A  E  F  E 200

601 GAATTGACCACCGACAATGTCTCGCCTTACACTCCTGGCGTTGCCTCTTCCTCTGAAGCT 660
201  E  L  T  T  D  N  V  S  P  Y  T  P  G  V  A  S  S  S  E  A 220

661 CCTGTTGCTATCCTTGGTGCCCGTGAATACATTTTCTCAGAGAACATTGGTGTACTTGGT 720
221  P  V  A  I  L  G  A  R  E  Y  I  F  S  E  N  I  G  V  L  G 240

721 GACGTTGCCGCCGGTAAAGAACAGACATTTGGTACCCTGTTTGCTCGTACTCTTGCTCAG 780
241  D  V  A  A  G  K  E  Q  T  F  G  T  L  F  A  R  T  L  A  Q 260

781 ATTGGCGGAAAGCTCCATTATGGTCACCCTGATTTCCTGAATGGTATCTTCATGACTACC 840
261  I  G  G  K  L  H  Y  G  H  P  D  F  L  N  G  I  F  M  T  T 280
```

FIG.5A

841 AGAGGTGGTATCTCCAAGGCTCAAAAAGGTCTACACCTTAACGAGGATATCTACGCTGGT 900
281 R G G I S K A Q K G L H L N E D I Y A G 300

901 ATGAACGCCATGGTTCGTGGTGGCCGCATCAAGCACTGCGAGTACTTCCAGTGTGGTAAG 960
301 M N A M V R G G R I K H C E Y F Q C G K 320

961 GGTCGTGATCTTGGTTTCGGTTCCATTCTTAATTTCACCACTAAGATTGGCACTGGTATG 1020
321 G R D L G F G S I L N F T T K I G T G M 340

1021 GGTGAGCAAATGCTATCAAGAGAGTACTACTACTKGGGTACTCAACTGCCACTCGACCGA 1080
341 G E Q M L S R E Y Y Y X G T Q L P L D R 360

1081 TTCCTGTCCTTTTACTATGYTCACCCTGGATTCCACATCAACAACATGTTTATTATGTTG 1140
361 F L S F Y Y X H P G F H I N N M F I M L 380

1141 TCTGTGCAAATGTTCATGATTGTTCTGATCAACCTGGGGGCCCTGAAGCACGAAACCATC 1200
381 S V Q M F M I V L I N L G A L K H E T I 400

1201 AACTGCAACTACAACTCCGACCTGCCCATTACCGATCCACTTATGCCAACGTTCTGCGCG 1260
401 N C N Y N S D L P I T D P L M P T F C A 420

1261 CCTCTCACTCCTATCATCAACTGGGTCAACCGCTGTGTTATTTCGATTTTCATCGTTTTC 1320
421 P L T P I I N W V N R C V I S I F I V F 440

1321 TTCATTTCGTTTGTTCCTTTGGCTGTTCAAGAATTGACTGAAAGAGGACTCTGGCGTATG 1380
441 F I S F V P L A V Q E L T E R G L W R M 460

1381 GCAACGCGTCTGGCCAAACATTTCGGATCTTTCTCCTTCATGTTCGAGGTGTTTGTTTGT 1440
461 A T R L A K H F G S F S F M F E V F V C 480

1441 CAAATCTATTCCAACGCTGTGCACCAAAACTTGTCTTTCGGTGGAGCGCGCTACATCGCT 1500
481 Q I Y S N A V H Q N L S F G G A R Y I A 500

1501 ACCGGTCGTGGTTTCGCAACTGCTCGTATCCCATTCGGCGTTCTGTACTCTCGGTTTGCG 1560
501 T G R G F A T A R I P F G V L Y S R F A 520

1561 GGACCTTCAATTTACACCGGTTTCCGTCTGCTGATCATGCTGCTCTTCTCAACCTCAACT 1620
521 G P S I Y T G F R L L I M L L F S T S T 540

FIG.5B

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1621 ACCTGGACTGCCTCTCTCATTTGGTTCTGGGTCTCTCTTCTCGCCCTTTGCATCTCCCCA 1680
541  T  W  T  A  S  L  I  W  F  W  V  S  L  L  A  L  C  I  S  P  560

1681 TTCCTTTTCAACCCTCACCAGTTTGCCTGGAACGACTTCTTCATCGATTACCGTGACTAC 1740
561  F  L  F  N  P  H  Q  F  A  W  N  D  F  F  I  D  Y  R  D  Y  580

1741 ATCCGATGGCTTTCGCGCGGTAACTCTCCCTCACACGCATCCTCATGGATTGGCTTCTGC 1800
581  I  R  W  L  S  R  G  N  S  R  S  H  A  S  S  W  I  G  F  C  600

1801 CGTTTGTCGCGTACTCGGATCACTGGTTACAAGCGCAAGCTTCTCGGTGTGCCGTCGGAG 1860
601  R  L  S  R  T  R  I  T  G  Y  K  R  K  L  L  G  V  P  S  E  620

1861 AAAGGATCAGGTGACGTTCCCAGAGCTCGTATTACCAACATTTTCTTCAGCGAAATTGTC 1920
621  K  G  S  G  D  V  P  R  A  R  I  T  N  I  F  F  S  E  I  V  640

1921 GCTCCTCTAGTCCTCGTTGCTGTTACCCTCGTTCCATACCTCTACATCAATTCTCGGACT 1980
641  A  P  L  V  L  V  A  V  T  L  V  P  Y  L  Y  I  N  S  R  T  660

1981 GGTGTGAGCGCTGATGTGGACGGGGGCAATGACCCTCACGATGCCATTTTGCGTATTGCC 2040
661  G  V  S  A  D  V  D  G  G  N  D  P  H  D  A  I  L  R  I  A  680

2041 ATTGTAGCATTTGGACCTATTGGTATCAATGCCGGTGTTGCTGCTGTTTTCTTTGGTATG 2100
681  I  V  A  F  G  P  I  G  I  N  A  G  V  A  A  V  F  F  G  M  700

2101 GCATGCTGCATGGGTCCCATCCTGAGCATGTGCTGCAAGAAGTTCGGTGCTGTGTTGGCG 2160
701  A  C  C  M  G  P  I  L  S  M  C  C  K  K  F  G  A  V  L  A  720

2161 GCTATTGCCCACGCGATTGCTGTGATCATCTTGCTTGTCATCTTTGAAGTCATGTTCTTC 2220
721  A  I  A  H  A  I  A  V  I  I  L  L  V  I  F  E  V  M  F  F  740

2221 CTCGAACACTGGTCTTGGCCCCGGTGCGTCATGGGCATGATCGCCATGGGTGCCATTCAA 2280
741  L  E  H  W  S  W  P  R  C  V  M  G  M  I  A  M  G  A  I  Q  760

2281 CGTTTCGTCTACAAACTTATTATCGCGCTCGCTCTTACCCGAGAGTTCAAGCATGACCAG 2340
761  R  F  V  Y  K  L  I  I  A  L  A  L  T  R  E  F  K  H  D  Q  780

2341 TCGAACATCGCATGGTGGACTGGAAAATGGTACAACATGGGTTGGGACTCTCTCTCTCAA 2400
781  S  N  I  A  W  W  T  G  K  W  Y  N  M  G  W  D  S  L  S  Q  800
```

FIG.5C

2401 CCGGGCCGAGAGTTCCTCTGCAAGATCACGGAGTTGGGCTATTTCTCAGCAGACTTCGTC 2460
801 P G R E F L C K I T E L G Y F S A D F V 820

2461 ATTGGTCATCTCCTATTGTTCATTATGCTGCCCGCTCTTTGTGTTCCTTACATTGACAAG 2520
821 I G H L L L F I M L P A L C V P Y I D K 840

2521 TTTCACTCAGYCATTCTCTTTTGGGTCCSGCCCAAGGTAAGAACC 2565
841 F H S X I L F W V X P K V R T 855

FIG.5D

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  1 GCATGCAAAC ATCTACACAA TTAGCAAGGG CAATCCATAT TTTGTCTTTT
 51 CGCGCCCTGG AAAGGCCTAA GTAATGTCGT AAACGCATTC TATCTGTACT
101 TCAACTCTCC TCTGTGCATT GGTTTGTGCA AATCACATTT TACGATACTG
151 CCAGATTTAT GCAAAAAGAG AAAACCAAGG GACCAGAACA AAGCAAAATT
201 ACGATAAACT TCGAATTCCT TCGTGCTTGA CTAAGACAAA GGGATGGACG
251 TAGCGATTTT TAGCGGGCCA AGAACTGGTT CCGAAAAAGC ACAGGTACAC
301 CGAACCCTCA GCTAAGGAGG GACAGCACCG ATGCGGAAGG ACAAACTTTC
351 TTTTTGCCTA TCACAGTATC TTATCGAGCT AACTATTTTC GACACACATG
401 AAAAAGCAGA AATATTAACG AAAAAGAAAA GAAAGACCAT GTCATGTACG
451 GGCAATCAGA ATCTGTAACA AGCGCCATTT TTTTTTCTGT ATCGGGCCCT
501 CCTTACTGCT CTCCTTCCGT GTAACGCGTT ATGAAACTCT AATCCTACTA
551 TCGGCGACTC TCTCGAAATT TTTCTTAACG CGTCCTTGTA CTGCGTCTAA
601 CGCTTTTGCC ACTTGGATTT CTATTATAGG AAATAGTCTC ACTTACTGGG
```

FIG. 6A

```
651   CGACGAATTT TCGCGTTTTG ATGAAGCACA GGAAGAATTT CTTTTTTTTT
701   TGGCTTCTTC TGGTTCCGTT TTTTACGCGC ACAAATCTAA AAAAAGAAAT
751   AATTATAACC TAGTCTCGAA AATTTTCATC GATCCATTCG TTCCTTTTTT
801   TCGATTTTTT CAGATCAAAA TTCTTGTTTC TTTCTTTGTC TTAGTTTATA
851   TTAAAAGATA TTTTGATTTT ACTCCTGAAC TATTTATTCT TTCTAAGAAG
901   GCCAGAACAC TACAGCTGTT TTAACCGACT ACGAAGTTCT CCATTCTCGA
951   ACACTAGCCT TCATTTACCA AACAGGAACT AGCGTATATC ATTAGTCCTT
1001  ATTCGAAAAG AGATTGGTAG ATATTTATTG TAGTTTGTGA GAAGGAGAAA
1051  ATACTGTCAT TGGACTGATA GTTAGAGGAC ATTAACCTCT CTTACGTTCG
1101  CTCAAAAAAA TTAAAATAAG CAAGTAGCTG AAATCAAGTC TTTCATACAA
1151  CGGTCAGACC ATGAACACTG ATCAACAACC TTATCAGGGC CAAACGGACT
1201  ATACCCAGGG ACCAGGTAAC GGGCAAAGTC AGGAACAAGA CTATGACCAA
1251  TATGGCCAGC CTTTGTATCC TTCACAAGCT GATGGTTACT ACGATCCAAA
```

FIG. 6B

1301	TGTCGCTGCT	GGTACTGAAG	CTGATATGTA	TGGTCAACAA	CCACCAAACG
1351	AGTCTTACGA	CCAAGACTAC	ACAAACGGTG	AATACTATGG	TCAACCGCCA
1401	AATATGGCTG	CTCAAGACGG	TGAAAACTTC	TCGGATTTTA	GCAGTTACGG
1451	CCCTCCTGGA	ACACCTGGAT	ATGATAGCTA	TGGTGGTCAG	TATACCGCTT
1501	CTCAAATGAG	TTATGGAGAA	CCAAATTCGT	CGGGTACCTC	GACTCCAATT
1551	TACGGTAATT	ATGACCCAAA	TGCTATCGCT	ATGGCTTTGC	CAAATGAACC
1601	TTATCCCGCT	TGGACTGCTG	ACTCTCAATC	TCCCGTTTCG	ATCGAGCAAA
1651	TCGAAGATAT	CTTTATTGAT	TTGACCAACA	GACTCGGGTT	CCAAAGAGAC
1701	TCCATGAGAA	ATATGTTTGA	TCATTTTATG	GTTCTCTTGG	ACTCTAGGTC
1751	CTCGAGAATG	TCTCCTGATC	AAGCTTTACT	ATCTTTACAT	GCCGACTACA
1801	TTGGTGGCGA	TACTGCTAAC	TATAAAAAAT	GGTATTTTGC	TGCTCAGTTA
1851	GATATGGATG	ATGAAATTGG	TTTTAGAAAT	ATGAGTCTTG	GAAAACTCTC
1901	AAGGAAGGCA	AGAAAAGCTA	AGAAGAAAAA	CAAGAAAGCA	ATGGAAGAGG

FIG. 6C

```
1951 CCAATCCCGA AGACACTGAA GAAACTTTAA ACAAAATTGA AGGCGACAAC
2001 TCCCTAGAGG CTGCTGATTT TAGATGGAAG GCCAAGATGA ACCAGTTGTC
2051 TCCCCTGGAA AGAGTTCGTC ATATCGCCTT ATATCTGTTA TGTTGGGGTG
2101 AAGCTAATCA AGTCAGATTC ACTGCTGAAT GTTTATGTTT TATCTACAAG
2151 TGTGCTCTTG ACTACTTGGA TTCCCCTCTT TGCCAACAAC GCCAAGAACC
2201 TATGCCAGAA GGTGATTTCT TGAATAGAGT CATTACGCCA ATTTATCATT
2251 TCATCAGAAA TCAAGTTTAT GAAATTGTTG ATGGTCGTTT TGTCAAGCGT
2301 GAAAGAGATC ATAACAAAAT TGTCGGTTAT GATGATTTAA ACCAATTGTT
2351 CTGGTATCCA GAAGGTATTG CAAAGATTGT TCTTGAAGAT GGAACAAAAT
2401 TGATAGAACT CCCATTGGAA GAACGTTATT TAAGATTAGG CGATGTCGTC
2451 TGGGATGATG TATTCTTCAA AACATATAAA GAGACCCGTA CTTGGTTACA
2501 TTTGGTCACC AACTTCAACC GTATTTGGGT TATGCATATC TCCATTTTTT
2551 GGATGTACTT TGCATATAAT TCACCAACAT TTTACACTCA TAACTATCAA
```

FIG. 6D

2601	CAATTGGTCG	ACAACCAACC	TTTGGCTGCT	TACAAGTGGG	CATCTTGCGC
2651	ATTAGGTGGT	ACTGTCGCAA	GTTTGATTCA	AATTGTCGCT	ACTTTGTGTG
2701	AATGGTCATT	CGTTCCAAGA	AAATGGGCTG	GTGCTCAACA	TCTATCTCGT
2751	AGATTCTGGT	TTTTATGCAT	CATCTTTGGT	ATTAATTTGG	GTCCTATTAT
2801	TTTTGTTTTT	GCTTACGACA	AAGATACAGT	CTACTCCACT	GCTGCACACG
2851	TTGTTGCTGC	TGTTATGTTC	TTTGTTGCGG	TTGCTACCAT	CATATTCTTC
2901	TCCATTATGC	CATTGGGGGG	GTTGTTTACG	TCATATATGA	AAAAATCTAC
2951	AAGGCGTTAT	GTTGCATCTC	AAACATTCAC	TGCTGCATTT	GCCCCTCTAC
3001	ATGGGTTAGA	TAGATGGATG	TCCTATTTAG	TTTGGGTTAC	TGTTTTTGCT
3051	GCCAAATATT	CAGAATCGTA	CTACTTTTTA	GTTTTATCTT	TGAGAGATCC
3101	AATTAGAATT	TTGTCCACCA	CTGCAATGAG	GTGTACAGGT	GAATACTGGT
3151	GGGGTGCGGT	ACTTTGTAAA	GTGCAACCCA	AGATTGTCTT	AGGTTTGGTT
3201	ATCGCTACCG	ACTTCATTCT	TTTCTTCTTG	GATACCTACT	TATGGTACAT

FIG. 6E

```
3251  TATTGTGAAT ACCATTTTCT CTGTTGGGAA ATCTTTCTAT TTAGGTATTT
3301  CTATCTTAAC ACCATGGAGA AATATCTTCA CAAGATTGCC AAAAAGAATA
3351  TACTCCAAGA TTTTGGCTAC TACTGATATG GAAATTAAAT ACAAACCAAA
3401  GGTTTTGATT TCTCAAGTAT GGAATGCCAT CATTATTTCA ATGTACAGAG
3451  AACATCTCTT AGCCATCGAC CATGTACAAA AATTACTATA TCATCAAGTT
3501  CCATCTGAAA TCGAAGGTAA AAGAACTTTG AGAGCTCCTA CCTTCTTTGT
3551  TTCTCAAGAT GACAATAATT TTGAGACTGA ATTTTTCCCT AGGGATTCAG
3601  AGGCTGAGCG TCGTATTTCT TTCTTTGCTC AATCTTTGTC TACTCCAATT
3651  CCCGAACCAC TTCCAGTTGA TAACATGCCA ACGTTCACAG TATTGACTCC
3701  TCACTACGCG GAAAGAATTC TGCTGTCATT AAGAGAAATT ATTCGTGAAG
3751  ATGACCAATT TTCTAGAGTT ACTCTTTTAG AATATCTAAA ACAATTACAT
3801  CCCGTTGAAT GGGAATGTTT TGTTAAGGAT ACTAAGATTT TGGCTGAAGA
3851  AACCGCTGCC TATGAAGGAA ATGAAAATGA AGCTGAAAAG GAAGATGCTT
```

FIG. 6F

3901	TGAAATCTCA	AATCGATGAT	TTGCCATTTT	ATTGTATTGG	TTTTAAATCT
3951	GCTGCTCCAG	AATATACACT	TCGTACGAGA	ATTTGGGCTT	CTTTGAGGTC
4001	GCAGACTCTA	TATCGTACCA	TTTCAGGGTT	CATGAATTAT	TCAAGAGCTA
4051	TCAAATTACT	GTATCGTGTG	GAAAATCCTG	AAATTGTTCA	AATGTTTGGT
4101	GGTAATGCTG	AAGGCTTAGA	AAGAGAGCTA	GAAAAGATGG	CAAGAAGAAA
4151	GTTTAAATTT	TTGGTCTCTA	TGCAGAGATT	GGCTAAATTC	AAACCACATG
4201	AACTGGAAAA	TGCTGAGTTT	TTGTTGAGAG	CTTACCCAGA	CTTACAAATT
4251	GCCTACTTGG	ATGAAGAGCC	ACCTTTGACT	GAAGGTGAGG	AGCCAAGAAT
4301	CTATTCCGCT	TTGATTGATG	GACATTGTGA	AATTCTAGAT	AATGGTCGTA
4351	GACGTCCCAA	GTTTAGAGTT	CAATTATCTG	GTAACCCAAT	TCTTGGTGAC
4401	GGTAAATCTG	ATAACCAAAA	CCATGCTTTG	ATTTTTTACA	GAGGTGAATA
4451	CATTCAATTA	ATTGATGCCA	ACCAAGATAA	CTACTTGGAA	GAATGTCTGA
4501	AGATTAGATC	TGTATTGGCT	GAATTTGAGG	AATTGAACGT	TGAACAAGTT

FIG. 6G

4551 AATCCATATG CTCCCGGTTT AAGGTATGAG GAGCAAACAA CTAATCATCC
4601 TGTTGCTATT GTTGGTGCCA GAGAATACAT TTTCTCTGAA AACTCTGGTG
4651 TGCTGGGTGA TGTGGCCGCT GGTAAAGAAC AAACTTTTGG TACATTATTT
4701 GCGCGTACTT TATCTCAAAT TGGTGGTAAA TTGCATTATG GTCATCCGGA
4751 TTTCATTAAT GCTACGTTTA TGACCACTAG AGGTGGTGTT TCCAAAGCAC
4801 AAAAGGGTTT GCATTTAAAC GAAGATATTT ATGCTGGTAT GAATGCTATG
4851 CTTCGTGGTG GTCGTATCAA GCATTGTGAG TATTATCAAT GTGGTAAAGG
4901 TAGAGATTTG GGTTTCGGTA CAATTCTAAA TTTCACTACT AAGATTGGTG
4951 CTGGTATGGG TGAACAAATG TTATCTCGTG AATATTATTA TCTGGGTACC
5001 CAATTACCAG TGGACCGTTT CCTAACATTC TATTATGCCC ATCCTGGTTT
5051 CCATTTGAAC AACTTGTTCA TTCAATTATC TTTGCAAATG TTTATGTTGA
5101 CTTTGGTGAA TTTATCTTCC TTGGCCCATG AATCTATTAT GTGTATTTAC
5151 GATAGGAACA AACCAAAAAC AGATGTTTTG GTTCCAATTG GGTGTTACAA

FIG. 6H

5201 CTTCCAACCT GCGGTTGATT GGGTGAGACG TTATACATTG TCTATTTTCA

5251 TTGTTTTCTG GATTGCCTTC GTTCCTATTG TTGTTCAAGA ACTAATTGAA

5301 CGTGGTCTAT GGAAAGCCAC CCAAAGATTT TTCTGCCACC TATTATCATT

5351 ATCCCCTATG TTCGAAGTGT TTGCGGGCCA AATCTACTCT TCTGCGTTAT

5401 TAAGTGATTT AGCAATTGGT GGTGCTCGTT ATATATCCAC CGGTCGTGGT

5451 TTTGCAACTT CTCGTATACC ATTTTCAATT TTGTATTCAA GATTTGCAGG

5501 ATCTGCTATC TACATGGGTG CAAGATCAAT GTTAATGTTG CTGTTCGGTA

5551 CTGTCGCACA TTGGCAAGCT CCACTACTGT GGTTTTGGGC CTCTCTATCT

5601 TCATTAATTT TTGCGCCTTT CGTTTTCAAT CCACATCAGT TTGCTTGGGA

5651 AGATTTCTTT TTGGATTACA GGGATTATAT CAGATGGTTA TCAAGAGGTA

5701 ATAATCAATA TCATAGAAAC TCGTGGATTG GTTACGTGAG GATGTCTAGG

5751 GCACGTATTA CTGGGTTTAA ACGTAAACTG GTTGGCGATG AATCTGAGAA

5801 AGCTGCTGGT GACGCAAGCA GGGCTCATAG AACCAATTTG ATCATGGCTG

FIG. 6I

```
5851  AAATCATACC CTGTGCAATT TATGCAGCTG GTTGTTTTAT TGCCTTCACG
5901  TTTATTAATG CTCAAACCGG TGTCAAGACT ACTGATGATG ATAGGGTGAA
5951  TTCTGTTTTA CGTATCATCA TTTGTACCTT GGCGCCAATC GCCGTTAACC
6001  TCGGTGTTCT ATTCTTCTGT ATGGGTATGT CATGCTGCTC TGGTCCCTTA
6051  TTTGGTATGT GTTGTAAGAA GACAGGTTCT GTAATGGCTG GAATTGCCCA
6101  CGGTGTTGCT GTTATTGTCC ACATTGCCTT TTTCATTGTC ATGTGGGTTT
6151  TGGAGAGCTT CAACTTTGTT AGAATGTTAA TCGGAGTCGT TACTTGTATC
6201  CAATGTCAAA GACTCATTTT TCATTGCATG ACAGCGTTAA TGTTGACTCG
6251  TGAATTTAAA AACGATCATG CCAATACAGC CTTCTGGACT GGTAAGTGGT
6301  ATGGTAAAGG TATGGGTTAC ATGGCTTGGA CCCAGCCAAG TAGAGAATTA
6351  ACCGCCAAGG TAATTGAGCT TTCAGAATTT GCAGCTGATT TTGTTCTAGG
6401  TCATGTGATT TTAATCTGTC AACTGCCACT CATTATAATC CCAAAAATAG
6451  ATAAATTCCA CTCGATTATG CTATTCTGGC TAAAGCCCTC TCGTCAAATT
```

FIG. 6J

6501	CGTCCCCCAA	TTTACTCTCT	GAAGCAAACT	CGTTTGCGTA	AGCGTATGGT
6551	CAAGAAGTAC	TGCTCTTTGT	ACTTTTTAGT	ATTGGCTATT	TTTGCAGGAT
6601	GCATTATTGG	TCCTGCTGTA	GCCTCTGCTA	AGATCCACAA	ACACATTGGA
6651	GATTCATTGG	ATGGCGTTGT	TCACAATCTA	TTCCAACCAA	TAAATACAAC
6701	CAATAATGAC	ACTGGTTCCC	AAATGTCAAC	TTATCAAAGT	CACTACTATA
6751	CTCATACGCC	ATCATTAAAG	ACCTGGTCAA	CTATAAAATA	ATACAATCAA
6801	TACTTGCTTG	AACGCTTGAT	TTTACTGATA	TTCTATCCAA	AAGCAAGTAG
6851	ACCAGAAACT	CTCAAGATGT	TGCAAATACC	GTTCGATGTT	TTTGGTTTAG
6901	ATTGTTTTAA	TGTTGATGCT	TTTTTACTTA	TTTTTGGAAG	CGTCTTTTTA
6951	ATTTAGTTTT	ATATTATAGG	TATATGAATG	TGTTTATGCC	AATAAGGGTT
7001	TTTTTGTACA	GTTATGTGAT	TATAAACAGT	CTTTTGTCTA	GTTTTTTTCA
7051	CCAGTATCGG	CCTCTATTTA	TAAAAAACGG	AGCAGCTTTC	GGTGTCAGTA
7101	ATTCTGAAAA	AATTTGTGTC	ACTCTGATTG	TAAATGAATT	AATTTAGCTA

FIG. 6K

7151 GATAGTTGCG AGCCCCAACG AGAAGATTGT CAGACAAAGA CAACATTCAA
7201 CAACCTACAT CCGTTACTAT TCGTTAACTC GAGGTACTTG AAACTTTTCA
7251 GTTAAGTATG AACAAGAAAC AAAATTTTTA CGCAGCCATT ATTGTGGCTA
7301 TTTTTCTTTG TTTGCAATTG TCTCATGGCT CTTCAGGTGT CAGCTTTGAA
7351 AAAACCCCTG CTATTAAAAT TGTAGGAAAC AAATTCTTTG ACTCTGAGAG
7401 TGGGGAACAG TTCTTCATCA AGGGCATTGC TTACCAATTG CAGAGAAGTG
7451 AAGAGGAGCT TAGCAATGCA AATGGGGCTT TTGAGACAAG TTATATTGAT
7501 GCCTTAGCGG ACCCAAAAAT ATGCTTAAGA GATATTCCAT TTTTGAAAAT
7551 GCTAGGAGTG AACACACTGC GTGTTTATGC AATAGATCCG ACAAAATCAC
7601 ATGATATATG TATGGAAGCT CTATCTGCCG AAGGAATGTA CGTCCTATTA
7651 GATCT

FIG. 6L

```
  1 MNTDQQPYQG QTDYTQGPGN GQSQEQDYDQ YGQPLYPSQA DGYYDPNVAA
 51 GTEADMYGQQ PPNESYDQDY TNGEYYGQPP NMAAQDGENF SDFSSYGPPG
101 TPGYDSYGGQ YTASQMSYGE PNSSGTSTPI YGNYDPNAIA MALPNEPYPA
151 WTADSQSPVS IEQIEDIFID LTNRLGFQRD SMRNMFDHFM VLLDSRSSRM
201 SPDQALLSLH ADYIGGDTAN YKKWYFAAQL DMDDEIGFRN MSLGKLSRKA
251 RKAKKKNKKA MEEANPEDTE ETLNKIEGDN SLEAADFRWK AKMNQLSPLE
301 RVRHIALYLL CWGEANQVRF TAECLCFIYK CALDYLDSPL CQQRQEPMPE
351 GDFLNRVITP IYHFIRNQVY EIVDGRFVKR ERDHNKIVGY DDLNQLFWYP
401 EGIAKIVLED GTKLIELPLE ERYLRLGDVV WDDVFFKTYK ETRTWLHLVT
451 NFNRIWVMHI SIFWMYFAYN SPTFYTHNYQ QLVDNQPLAA YKWASCALGG
501 TVASLIQIVA TLCEWSFVPR KWAGAQHLSR RFWFLCIIFG INLGPIIFVF
551 AYDKDTVYST AAHVVAAVMF FVAVATIIFF SIMPLGGLFT SYMKKSTRRY
601 VASQTFTAAF APLHGLDRWM SYLVWVTVFA AKYSESYYFL VLSLRDPIRI
```

FIG. 7A

651	LSTTAMRCTG	EYWWGAVLCK	VQPKIVLGLV	IATDFILFFL	DTYLWYIIVN
701	TIFSVGKSFY	LGISILTPWR	NIFTRLPKRI	YSKILATTDM	EIKYKPKVLI
751	SQVWNAIIIS	MYREHLLAID	HVQKLLYHQV	PSEIEGKRTL	RAPTFFVSQD
801	DNNFETEFFP	RDSEAERRIS	FFAQSLSTPI	PEPLPVDNMP	TFTVLTPHYA
851	ERILLSLREI	IREDDQFSRV	TLLEYLKQLH	PVEWECFVKD	TKILAEETAA
901	YEGNENEAEK	EDALKSQIDD	LPFYCIGFKS	AAPEYTLRTR	IWASLRSQTL
951	YRTISGFMNY	SRAIKLLYRV	ENPEIVQMFG	GNAEGLEREL	EKMARRKFKF
1001	LVSMQRLAKF	KPHELENAEF	LLRAYPDLQI	AYLDEEPPLT	EGEEPRIYSA
1051	LIDGHCEILD	NGRRRPKFRV	QLSGNPILGD	GKSDNQNHAL	IFYRGEYIQL
1101	IDANQDNYLE	ECLKIRSVLA	EFEELNVEQV	NPYAPGLRYE	EQTTNHPVAI
1151	VGAREYIFSE	NSGVLGDVAA	GKEQTFGTLF	ARTLSQIGGK	LHYGHPDFIN
1201	ATFMTTRGGV	SKAQKGLHLN	EDIYAGMNAM	LRGGRIKHCE	YYQCGKGRDL
1251	GFGTILNFTT	KIGAGMGEQM	LSREYYYLGT	QLPVDRFLTF	YYAHPGFHLN

FIG. 7B

```
1301  NLFIQLSLQM FMLTLVNLSS LAHESIMCIY DRNKPKTDVL VPIGCYNFQP
1351  AVDWVRRYTL SIFIVFWIAF VPIVVQELIE RGLWKATQRF FCHLLSLSPM
1401  FEVFAGQIYS SALLSDLAIG GARYISTGRG FATSRIPFSI LYSRFAGSAI
1451  YMGARSMLML LFGTVAHWQA PLLWFWASLS SLIFAPFVFN PHQFAWEDFF
1501  LDYRDYIRWL SRGNNQYHRN SWIGYVRMSR ARITGFKRKL VGDESEKAAG
1551  DASRAHRTNL IMAEIIPCAI YAAGCFIAFT FINAQTGVKT TDDDRVNSVL
1601  RIIICTLAPI AVNLGVLFFC MGMSCCSGPL FGMCCKKTGS VMAGIAHGVA
1651  VIVHIAFFIV MWVLESFNFV RMLIGVVTCI QCQRLIFHCM TALMLTREFK
1701  NDHANTAFWT GKWYGKGMGY MAWTQPSREL TAKVIELSEF AADFVLGHVI
1751  LICQLPLIII PKIDKFHSIM LFWLKPSRQI RPPIYSLKQT RLRKRMVKKY
1801  CSLYFLVLAI FAGCIIGPAV ASAKIHKHIG DSLDGVVHNL FQPINTTNND
1851  TGSQMSTYQS HYYTHTPSLK TWSTIK
```

FIG. 7C

```
  1 GAATTCCCCT CGCAACACTG AAAGATGCCA TTGTCAAAGG TGAAATTGCC
 51 GCGTGGCCCC TAGATCCTGC TCGTGAACGA TGGACGCGGC CTGCGCTATT
101 CATCAGGGCT ACTCAATCGC ATTATGTGGT AGACGAGTAT CTTCCGATCA
151 TCGGCGCGTT CTTTCCACGC TTTGAAACAC GTGACATCGA TGCGGGTCAC
201 TGGGTAAATG CGGAGAAGCC TGGGGAATGT GCCGAAAGCA TCGTCGATTT
251 TGTGGAGCGG CACGAGGATT AAAGGCAAGC GCCCCGGAGC AAGGTGCCAG
301 TAGCACCAGT CGGTGGCTGT GCGCTTGCCG TAGCACATGA CATACGGACT
351 ATTGTGTGAG TGGTGATGGG GTGTAGGCAG TGCCACACCA GTTTAAAGGC
401 CTAGTAACGG CAAATCGCCA AAAGAGATGA TGCTGATGCA TACGATAAGA
451 TCGTCAGTTT CACGTTCGCG GTTCGAACAT GGAATTGTGG CTAAAGAAAT
501 TTGGGCGGTA TGATGCAAAT GAGGTGTACG TATGTATATA TAGCAAAGAG
551 TAGAATAAAA TGAGATAAAG CCTCGTTCGT TCTCTCCATT TCTTCCCTGT
601 TTCTCCTTTA TTTTCTCTAC TGCTTATTTC GAGTTCACCA GAGAACAAGA
```

FIG. 8A

```
651  GAGCAGGAAC GCAAAGAGTG TGTGACACGA AATTCAAGAT ACAAAAATAA
701  AAGCTTACGT TGTGTATTTC AACTGGTGTG CTAAGAATAG AGTTTCATAA
751  AGTACTGCAT TTATTCATAT ATTATTTTTG TTATTTGTAT ATATACTTCA
801  CACTTAGAGT TCTACTAAAA GTCTACCCAG CACGCATCCT TCGTTTATTT
851  TTACATCTCT CTTTTGCTTT TCCTTTTTTT TTTTGGTGCT TGCTAGATAC
901  TACTGAAGAT CAAAGGTTAC AAAGAACGCC GCATATATTT TCTGCAGGCA
951  TATTAAAGAA GTTACAAAAG GATTAATCGA AGCGCTGTTT GGATACACTC
1001 CTGTAAAGAG AAGAAAAGGA AAAAAATAAA AAGTGGACAA TAAATAATTA
1051 TTAAACTGTC ATAGTTATGT CCTACAACGA TCCAAACTTG AATGGACAGT
1101 ATTACAGTAA CGGTGATGGG ACTGGTGACG GTAATTACCC TACGTACCAA
1151 GTGACACAGG ATCAAAGTGC GTACGATGAG TACGGTCAGC CAATCTATAC
1201 ACAAAACCAA CTGGATGATG GTTATTATGA TCCAAACGAA CAATACGTTG
1251 ACGGTACACA ATTTCCTCAG GGACAAGATC CTTCACAAGA CCAAGGTCCT
```

FIG. 8B

```
1301  TATAATAACG ATGCTAGTTA CTATAACCAA CCCCCCAATA TGATGAACCC
1351  GTCTTCTCAA GATGGAGAGA ACTTCTCAGA TTTTAGCAGC TATGGTCCCC
1401  CATCCGGCAC TTATCCTAAC GATCAATATA CTCCTTCTCA AATGAGTTAT
1451  CCTGATCAAG ATGGTTCTTC AGGGGCCTCA ACCCCCTATG GAAATGGTGT
1501  CGTTAATGGT AATGGCCAGT ACTACGACCC TAATGCTATT GAAATGGCTT
1551  TACCAAATGA TCCATATCCC GCATGGACCG CAGATCCCCA GTCTCCCCTG
1601  CCCATCGAAC AAATCGAAGA TATCTTCATA GATTTAACAA ATAAATTCGG
1651  TTTTCAGAGA GATTCCATGA GAAATATGTT TGACCATTTT ATGACCCTTT
1701  TGGACTCTAG ATCTTCTAGG ATGTCTCCAG AACAGGCCCT TTTATCATTA
1751  CATGCAGACT ACATAGGTGG AGATACGGCC AACTACAAAA AATGGTACTT
1801  TGCCGCTCAA CTTGATATGG ATGATGAAAT TGGTTTCAGG AATATGAAGT
1851  TGGGTAAGCT ATCAAGAAAG GCAAGAAAGG CTAAGAAGAA AAATAAAAAA
1901  GCCATGCAAG AGGCTAGTCC TGAAGACACT GAGGAGACTT TAAATCAAAT
```

FIG. 8C

1951	TGAGGGTGAT	AACTCATTAG	AAGCTGCGGA	TTTTAGATGG	AAGTCAAAGA
2001	TGAATCAACT	TTCTCCATTT	GAAATGGTTC	GTCAAATTGC	CTTGTTTTTA
2051	TTATGTTGGG	GCGAGGCAAA	TCAAGTCAGA	TTTACCCCGG	AGTGTCTTTG
2101	TTTCATTTAT	AAATGCGCCT	CTGATTACTT	AGATTCTGCA	CAATGTCAAC
2151	AACGTCCTGA	TCCCTTGCCT	GAAGGTGATT	TTTTGAATAG	AGTTATTACT
2201	CCTCTTTATC	GTTTTATTAG	GAGCCAGGTT	TACGAAATCG	TGGATGGTCG
2251	ATACGTGAAG	AGTGAAAAAG	ATCATAACAA	AGTTATTGGG	TATGATGATG
2301	TCAATCAATT	ATTCTGGTAT	CCAGAAGGTA	TAGCAAAAAT	TGTCATGGAA
2351	GATGGAACCA	GGTTGATTGA	TTTGCCAGCA	GAGGAGCGTT	ATTTGAAATT
2401	GGGAGAAATT	CCCTGGGATG	ATGTCTTCTT	TAAAACTTAC	AAAGAAACAC
2451	GTTCCTGGTT	ACATTTAGTT	ACCAACTTCA	ATCGTATTTG	GATCATGCAC
2501	ATCTCAGTAT	ATTGGATGTA	TTGTGCTTAC	AATGCTCCAA	CTTTTTATAC
2551	TCACAACTAT	CAACAATTGG	TCGACAATCA	GCCTTTGGCA	GCTTATAAAT

FIG. 8D

```
2601 GGGCCACTGC AGCATTAGGT GGTACTGTGG CAAGTTTGAT TCAAGTTGCC
2651 GCTACTTTGT GCGAGTGGTC ATTCGTTCCT AGAAAATGGG CGGGTGCTCA
2701 ACATTTGTCC CGTAGATTCT GGTTCTTGTG TGTCATTATG GGTATTAATT
2751 TGGGGCCTGT GATATTTGTT TTCGCTTATG ATAAGGACAC AGTATATTCT
2801 ACTGCCGCTC ATGTCGTTGG AGCAGTTATG TTTTTTGTTG CTGTGGCAAC
2851 ACTTGTTTTC TTTTCCGTAA TGCCATTGGG TGGATTATTT ACATCGTATA
2901 TGAAAAAGTC CACAAGAAGT TATGTTGCCT CACAGACCTT CACCGCATCT
2951 TTTGCTCCAT TGCATGGTTT AGACAGGTGG ATGTCTTATT TGGTTTGGGT
3001 AACCGTTTTT GCTGCTAAAT ATGCAGAGTC ATATTTTTTT CTAATACTGT
3051 CACTAAGAGA TCCAATTAGG ATTTTATCTA CTACATCAAT GAGATGTACT
3101 GGTGAATACT GGTGGGGTAA TAAGATTTGT AAGGTCCAGC CAAAGATTGT
3151 TTTAGGTTTA ATGATTGCGA CTGACTTCAT TTTGTTCTTT TTGGATACCT
3201 ACTTGTGGTA TATCGTTGTT AACACTGTTT TCTCGGTCGG AAAATCGTTC
```

FIG. 8E

```
3251  TATTTGGGTA TTTCTATCTT AACTCCATGG AGAAATATTT TCACTAGATT
3301  GCCAAAAAGA ATTTATTCTA AGATCTTGGC TACTACTGAT ATGGAAATAA
3351  AATATAAACC GAAAGTACTA ATTTCTCAGA TTTGGAATGC TATCATTATC
3401  TCCATGTACA GAGAACATTT ATTAGCCATA GACCATGTAC AAAAATTGTT
3451  ATATCATCAG GTTCCGTCCG AAATTGAAGG TAAGAGGACT TTGAGAGCAC
3501  CAACTTTCTT TGTTTCCCAA GATGACAATA ATTTTGAGAC TGAATTTTTC
3551  CCTAGAGATT CAGAAGCTGA GCGCCGTATT TCATTTTTTG CCCAATCTCT
3601  ATCCACTCCA ATTCCAGAAC CACTACCAGT TGACAACATG CCAACATTTA
3651  CTGTATTAAC TCCCCATTAC GCCGAGAGGA TTCTATTATC ATTGAGAGAA
3701  ATTATTCGTG AAGATGATCA ATTTTCAAGA GTTACTCTTT TGGAATACCT
3751  GAAGCAATTA CACCCGGTAG AATGGGACTG TTTTGTTAAG GATACGAAAA
3801  TTCTTGCTGA AGAAACGGCC GCATATGAAA ACAATGAAGA TGAACCTGAA
3851  AAGGAAGACG CTCTGAAATC TCAAATTGAT GATTTACCTT TCTATTGTAT
```

FIG. 8F

```
3901  TGGTTTCAAA TCTGCTGCAC CAGAATACAC CTTACGTACG AGAATCTGGG
3951  CCTCTTTAAG GTCGCAAACT TTGTATCGCA CAATCTCGGG GTTTATGAAT
4001  TATTCGAGGG CCATAAAATT ACTTTATCGT GTGGAAAATC CAGAAATCGT
4051  TCAAATGTTC GGTGGTAATG CTGATGGATT AGAAAGAGAA CTGGAAAAAA
4101  TGGCAAGGCG AAAATTCAAA TTCTTGGTTT CGATGCAAAG ATTGGCCAAG
4151  TTTAAACCAC ATGAACTAGA AAATGCTGAG TTCCTGTTGA GAGCTTATCC
4201  GGACTTGCAA ATTGCCTACC TGGATGAAGA ACCTCCCTTA AACGAAGGCG
4251  AAGAGCCAAG AATTTACTCG GCCTTAATTG ATGGTCATTG TGAGATTTTA
4301  GAGAATGGTC GTAGACGTCC CAAATTTAGA GTTCAACTAT CCGGTAATCC
4351  AATTCTTGGT GATGGTAAAT CAGATAATCA AAATCATGCT TTGATTTTTT
4401  ACAGAGGTGA GTATATTCAA TTGATTGATG CTAATCAAGA CAATTACTTG
4451  GAAGAGTGTT TGAAAATCAG GTCTGTCTTA GCAGAATTTG AAGAATTGGG
4501  AATTGAGCAA ATTCATCCTT ATACTCCTGG TTTAAAATAT GAGGACCAAT
```

FIG. 8G

```
4551  CCACAAATCA TCCTGTTGCA ATTGTCGGCG CTAGAGAATA TATTTTCTCA
4601  GAAAACTCTG GTGTTCTTGG TGATGTAGCG GCTGGTAAAG AACAAACTTT
4651  TGGTACATTA TTTGCCCGTA CTTTGGCACA GATTGGTGGT AAATTGCATT
4701  ATGGTCATCC AGATTTTATT AATGCGACAT TCATGACTAC TAGGGGTGGT
4751  GTTTCCAAAG CACAAAAGGG TCTACATTTA AATGAAGATA TTTATGCCGG
4801  TATGAATGCC GTACTTCGGG GTGGTCGTAT CAAGCATTGC GAATATTATC
4851  AGTGTGGTAA AGGTAGAGAT TTAGGTTTTG GTACAATTTT GAATTTCACT
4901  ACTAAGATCG GTGCTGGTAT GGGTGAACAA ATGTTATCTC GTGAATACTA
4951  TTATTTGGGT ACGCAATTAC CTATTGACCG TTTTTTAACA TTTTATTATG
5001  CGCATCCAGG GTTTCACTTG AATAACTTAT TTATTCAATT GTCTCTGCAG
5051  ATGTTCATGT TAACTTTAGT GAACTTGCAT GCTTTGGCTC ATGAATCCAT
5101  TCTGTGTGTT TACGATAGGG ATAAGCCAAT TACTGATGTT TTGTATCCAA
5151  TTGGTTGTTA CAACTTTCAT CCTGCGATTG ATTGGGTGAG ACGTTATACA
```

FIG. 8H

```
5201  CTCTCTATTT TCATCGTCTT TTGGATTGCT TTTGTCCCTA TTGTCGTTCA
5251  GGAATTAATC GAGCGTGGTC TGTGGAAGGC GACACAAAGA TTTTTCCGTC
5301  ACATTTTATC TCTATCTCCA ATGTTTGAAG TCTTTGCTGG CCAAATCTAT
5351  TCTTCAGCAC TGTTAAGTGA TATCGCTGTG GGTGGTGCTC GTTATATTTC
5401  AACAGGTCGT GGCTTTGCTA CATCTCGTAT ACCTTTCTCT ATTCTTTATT
5451  CAAGATTTGC GGGTTCAGCC ATTTATATGG GATCAAGATC AATGTTGATG
5501  TTATTATTTG GTACCGTGGC ACATTGGCAA GCTCCACTAT TATGGTTTTG
5551  GGCATCATTA TCAGCCTTAA TCTTTGCACC ATTCATTTTC AATCCACATC
5601  AATTTGCTTG GGAAGATTTT TTCCTAGACT ACAGAGATTA TATCAGATGG
5651  CTGTCAAGAG GTAATAATAA GTACCACAGG AACTCATGGA TTGGTTATGT
5701  AAGAATGTCG AGGTCTCGTG TTACTGGTTT CAAGCGCAAA CTGGTGGGTG
5751  ATGAGTCTGA AAAATCTGCA GGCGATGCAA GCAGGGCTCA TAGAACCAAT
5801  TTAATTATGG CTGAAATTAT ACCGTGTGCG ATTTACGCAG CAGGTTGTTT
```

FIG. 8I

5851 TATTGCCTTC ACGTTTATTA ATGCACAAAC TGGTGTCAAG ACTACTGATG
5901 AAGATAGAGT AAATTCCACC TTACGTATCA TCATTTGCAC CTTGGCGCCT
5951 ATTGTTATCG ATATTGGTGT TTTATTCTTC TGTATGGGTT TGTCATGCTG
6001 CTCTGGCCCT TTGTTGGGCA TGTGCTGCAA GAAAACTGGT TCTGTTATGG
6051 CAGGGATCGC TCACGGTATC GCTGTTGTTG TCCATATTGT CTTTTTCATT
6101 GTCATGTGGG TTTTAGAGGG TTTTAGTTTT GTTAGGATGT TGATTGGCGT
6151 TGTTACATGT ATACAATGTC AAAGGTTGAT TTTTCACTGT ATGACTGTAC
6201 TGTTGCTGAC CCGTGAGTTC AAGAATGATC ACGCTAATAC TGCCTTTTGG
6251 ACAGGCAAAT GGTACAGCAC CGGTTTAGGA TATATGGCAT GGACTCAACC
6301 GACAAGGGAA TTGACTGCAA AAGTCATTGA GCTTTCCGAG TTTGCAGCGG
6351 ATTTTGTTTT GGGGCATGTA ATTTTGATCT TCCAACTACC AGTCATTTGT
6401 ATTCCAAAGA TAGATAAGTT TCACTCCATC ATGTTATTTT GGTTAAAACC
6451 ATCCCGTCAA ATCCGTCCTC CTATTTACTC TTTGAAACAA GCACGCCTAC

FIG. 8J

```
6501  GTAAACGTAT GGTTAGGAGG TATTGCAGCT TGTACTTTTT GGTACTGATC
6551  ATATTCGCGG GATGCATCGT TGGCCCTGCC GTTGCTTCAG CACATGTTCC
6601  AAAAGACCTT GGATCTGGGT TGACGGGTAC TTTCCATAAC TTGGTTCAAC
6651  CAAGGAACGT ATCTAACAAT GATACAGGGT CCCAGATGTC TACTTATAAG
6701  AGTCATTATT ACACTCATAC GCCATCCTTA AAGACCTGGT CTACGATCAA
6751  ATGATTTTTT TAGTTTACAA TCTATTTTTG TTTCTAAGCA AGTTTATCAC
6801  GCAAATACAT AAGTATATTT TTACTTTCTA TTCTTCCTAG TTTATATTTA
6851  TTTCATTGTA ACTTTCTTAG AAGCTCGGTC CTCTCGCTAT ATAGTAGGAT
6901  CTGCAACATA TTTGGATGTG GGTGGGCGTT CTCCTTCTTT TTTAGATGTA
6951  AGGTCCAACA CGTATAACAG GTGATACACA TAGAAAGACA CGTGGAAATA
7001  ACAGTCATTT ACGAATATTT AAAACCTGAG CAACTCCGTC AAATTTGATC
7051  TTAATCTTTT CTGGGGCCCC
```

FIG. 8K

1	MSYNDPNLNG	QYYSNGDGTG	DGNYPTYQVT	QDQSAYDEYG	QPIYTQNQLD
51	DGYYDPNEQY	VDGTQFPQGQ	DPSQDQGPYN	NDASYYNQPP	NMMNPSSQDG
101	ENFSDFSSYG	PPSGTYPNDQ	YTPSQMSYPD	QDGSSGASTP	YGNGVVNGNG
151	QYYDPNAIEM	ALPNDPYPAW	TADPQSPLPI	EQIEDIFIDL	TNKFGFQRDS
201	MRNMFDHFMT	LLDSRSSRMS	PEQALLSLHA	DYIGGDTANY	KKWYFAAQLD
251	MDDEIGFRNM	KLGKLSRKAR	KAKKKNKKAM	QEASPEDTEE	TLNQIEGDNS
301	LEAADFRWKS	KMNQLSPFEM	VRQIALFLLC	WGEANQVRFT	PECLCFIYKC
351	ASDYLDSAQC	QQRPDPLPEG	DFLNRVITPL	YRFIRSQVYE	IVDGRYVKSE
401	KDHNKVIGYD	DVNQLFWYPE	GIAKIVMEDG	TRLIDLPAEE	RYLKLGEIPW
451	DDVFFKTYKE	TRSWLHLVTN	FNRIWIMHIS	VYWMYCAYNA	PTFYTHNYQQ
501	LVDNQPLAAY	KWATAALGGT	VASLIQVAAT	LCEWSFVPRK	WAGAQHLSRR
551	FWFLCVIMGI	NLGPVIFVFA	YDKDTVYSTA	AHVVGAVMFF	VAVATLVFFS
601	VMPLGGLFTS	YMKKSTRSYV	ASQTFTASFA	PLHGLDRWMS	YLVWVTVFAA

FIG. 9A

```
651   KYAESYFFLI LSLRDPIRIL STTSMRCTGE YWWGNKICKV QPKIVLGLMI
701   ATDFILFFLD TYLWYIVVNT VFSVGKSFYL GISILTPWRN IFTRLPKRIY
751   SKILATTDME IKYKPKVLIS QIWNAIIISM YREHLLAIDH VQKLLYHQVP
801   SEIEGKRTLR APTFFVSQDD NNFETEFFPR DSEAERRISF FAQSLSTPIP
851   EPLPVDNMPT FTVLTPHYAE RILLSLREII REDDQFSRVT LLEYLKQLHP
901   VEWDCFVKDT KILAEETAAY ENNEDEPEKE DALKSQIDDL PFYCIGFKSA
951   APEYTLRTRI WASLRSQTLY RTISGFMNYS RAIKLLYRVE NPEIVQMFGG
1001  NADGLERELE KMARRKFKFL VSMQRLAKFK PHELENAEFL LRAYPDLQIA
1051  YLDEEPPLNE GEEPRIYSAL IDGHCEILEN GRRRPKFRVQ LSGNPILGDG
1101  KSDNQNHALI FYRGEYIQLI DANQDNYLEE CLKIRSVLAE FEELGIEQIH
1151  PYTPGLKYED QSTNHPVAIV GAREYIFSEN SGVLGDVAAG KEQTFGTLFA
1201  RTLAQIGGKL HYGHPDFINA TFMTTRGGVS KAQKGLHLNE DIYAGMNAVL
1251  RGGRIKHCEY YQCGKGRDLG FGTILNFTTK IGAGMGEQML SREYYYLGTQ
```

FIG. 9B

```
1301 LPIDRFLTFY YAHPGFHLNN LFIQLSLQMF MLTLVNLHAL AHESILCVYD
1351 RDKPITDVLY PIGCYNFHPA IDWVRRYTLS IFIVFWIAFV PIVVQELIER
1401 GLWKATQRFF RHILSLSPMF EVFAGQIYSS ALLSDIAVGG ARYISTGRGF
1451 ATSRIPFSIL YSRFAGSAIY MGSRSMLMLL FGTVAHWQAP LLWFWASLSA
1501 LIFAPFIFNP HQFAWEDFFL DYRDYIRWLS RGNNKYHRNS WIGYVRMSRS
1551 RVTGFKRKLV GDESEKSAGD ASRAHRTNLI MAEIIPCAIY AAGCFIAFTF
1601 INAQTGVKTT DEDRVNSTLR IIICTLAPIV IDIGVLFFCM GLSCCSGPLL
1651 GMCCKKTGSV MAGIAHGIAV VVHIVFFIVM WVLEGFSFVR MLIGVVTCIQ
1701 CQRLIFHCMT VLLLTREFKN DHANTAFWTG KWYSTGLGYM AWTQPTRELT
1751 AKVIELSEFA ADFVLGHVIL IFQLPVICIP KIDKFHSIML FWLKPSRQIR
1801 PPIYSLKQAR LRKRMVRRYC SLYFLVLIIF AGCIVGPAVA SAHVPKDLGS
1851 GLTGTFHNLV QPRNVSNNDT GSQMSTYKSH YYTHTPSLKT WSTIK
```

FIG. 9C

1 TACTGTATCGGTTTCAAGTCTGCTGCTCCCGAGTACACGCTTCGCACCCGTATTTGGTCC
60
1 Y C I G F K S A A P E Y T L R T R I W S 20

61 TCGCTGCGTTCGCAAACTCTTTACAGAACTGTATCCGGGATGATGAACTATAGCAGAGCT
120
21 S L R S Q T L Y R T V S G M M N Y S R A 40

121 ATCAAGCTCCTCTACCGTGTGGAGAACCCGGAAGTCGTCCAGATGTTCGGTGGTAATTCT
180
41 I K L L Y R V E N P E V V Q M F G G N S 60

181
GAGAAGCTGGAACATGAGCTCGAGAGGATGGCCCGTCGCAAGTTCAAGATCTGTGTTTCA 240
61 E K L E H E L E R M A R R K F K I C V S 80

241
ATGCAGCGGTATGCCAAATTCACAAAAGAAGAACGTGAGAACACAGAGTTCCTCCTCCGA 300
81 M Q R Y A K F T K E E R E N T E F L L R 100

301
GCCTACCCCGACCTGCAGATTGCCTATCTCGATGAGGAACCTCCAGCCAACGAGGGTGAA 360
101 A Y P D L Q I A Y L D E E P P A N E G E 120

361 GAGCCGCGTCTCTACTCTGCTTTGATTGATGGACACTGTGAGCTGCTCGAGAATGGCATG
420
121 E P R L Y S A L I D G H C E L L E N G M 140

FIG. 10A

```
421
CGGAAGCCCAAGTTCAGGATCCAGCTCTCCGGAAACCCGATCCTTGGTGACGGCAAGTCT 480
 141 R K P K F R I Q L S G N P I L G D G K S  160

481
GACAACCAAAACCACTCGATCATTTTCTACCGCGGTGAATACATTCAGGTCATTGATGCC 540
 161 D N Q N H S I I F Y R G E Y I Q V I D A  180

541
AACCAAGACAACTATCTCGAAGAGTGCTTGAAAATCCGAAGCGTTCTTGCTGAGTTTGAG 600
 181 N Q D N Y L E E C L K I R S V L A E F E  200

 601 GAATTGACCACCGACAATGTCTCGCCTTACACTCCTGGCGTTGCCTCTTCCTCTGAAGCT
660
 201 E L T T D N V S P Y T P G V A S S S E A  220

 661 CCTGTTGCTATCCTTGGTGCCCGTGAATACATTTTCTCAGAGAACATTGGTGTACTTGGT
720
 221 P V A I L G A R E Y I F S E N I G V L G  240

 721 GACGTTGCCGCCGGTAAAGAACAGACATTTGGTACCCTGTTTGCTCGTACTCTTGCTCAG
780
 241 D V A A G K E Q T F G T L F A R T L A Q  260

781
ATTGGCGGAAAGCTCCATTATGGTCACCCTGATTTCCTGAATGGTATCTTCATGACTACC 840
 261 I G G K L H Y G H P D F L N G I F M T T  280
```

FIG. 10B

841
AGAGGTGGTATCTCCAAGGCTCAAAAAGGTCTACACCTTAACGAGGATATCTACGCTGGT 900
281 R G G I S K A Q K G L H L N E D I Y A G 300

901 ATGAACGCCATGGTTCGTGGTGGCCGCATCAAGCACTGCGAGTACTTCCAGTGTGGTAAG
960
301 M N A M V R G G R I K H C E Y F Q C G K 320

961 GGTCGTGATCTTGGTTTCGGTTCCATTCTTAATTTCACCACTAAGATTGGCACTGGTATG
1020
321 G R D L G F G S I L N F T T K I G T G M 340

1021
GGTGAGCAAATGCTATCAAGAGAGTACTACTACTKGGGTACTCAACTGCCACTCGACCGA
1080
341 G E Q M L S R E Y Y Y X G T Q L P L D R 360

1081
TTCCTGTCCTTTTACTATGYTCACCCTGGATTCCACATCAACAACATGTTTATTATGTTG 1140
361 F L S F Y Y X H P G F H I N N M F I M L 380

1141
TCTGTGCAAATGTTCATGATTGTTCTGATCAACCTGGGGGCCCTGAAGCACGAAACCATC 1200
381 S V Q M F M I V L I N L G A L K H E T I 400

1201
AACTGCAACTACAACTCCGACCTGCCCATTACCGATCCACTTATGCCAACGTTCTGCGCG 1260
401 N C N Y N S D L P I T D P L M P T F C A 420

FIG. 10C

1261
CCTCTCACTCCTATCATCAACTGGGTCAACCGCTGTGTTATTTCGATTTTCATCGTTTTC 1320
421 P L T P I I N W V N R C V I S I F I V F 440

1321
TTCATTTCGTTTGTTCCTTTGGCTGTTCAAGAATTGACTGAAAGAGGACTCTGGCGTATG 1380
441 F I S F V P L A V Q E L T E R G L W R M 460

1381 GCAACGCGTCTGGCCAAACATTTCGGATCTTTCTCCTTCATGTTCGAGGTGTTTGTTTGT
1440
461 A T R L A K H F G S F S F M F E V F V C 480

1441
CAAATCTATTCCAACGCTGTGCACCAAAACTTGTCTTTCGGTGGAGCGCGCTACATCGCT 1500
481 Q I Y S N A V H Q N L S F G G A R Y I A 500

1501 ACCGGTCGTGGTTTCGCAACTGCTCGTATCCCATTCGGCGTTCTGTACTCTCGGTTTGCG
1560
501 T G R G F A T A R I P F G V L Y S R F A 520

1561
GGACCTTCAATTTACACCGGTTTCCGTCTGCTGATCATGCTGCTCTTCTCAACCTCAACT 1620
521 G P S I Y T G F R L L I M L L F S T S T 540

1621 ACCTGGACTGCCTCTCTCATTTGGTTCTGGGTCTCTCTTCTCGCCCTTTGCATCTCCCCA
1680
541 T W T A S L I W F W V S L L A L C I S P 560

FIG. 10D

1681
TTCCTTTTCAACCCTCACCAGTTTGCCTGGAACGACTTCTTCATCGATTACCGTGACTAC 1740
561 F L F N P H Q F A W N D F F I D Y R D Y 580

1741 ATCCGATGGCTTTCGCGCGGTAACTCTCGCTCACACGCATCCTCATGGATTGGCTTCTGC
1800
581 I R W L S R G N S R S H A S S W I G F C 600

1801 CGTTTGTCGCGTACTCGGATCACTGGTTACAAGCGCAAGCTTCTCGGTGTGCCGTCGGAG
1860
601 R L S R T R I T G Y K R K L L G V P S E 620

1861
AAAGGATCAGGTGACGTTCCCAGAGCTCGTATTACCAACATTTTCTTCAGCGAAATTGTC 1920
621 K G S G D V P R A R I T N I F F S E I V 640

1921 GCTCCTCTAGTCCTCGTTGCTGTTACCCTCGTTCCATACCTCTACATCAATTCTCGGACT
1980
641 A P L V L V A V T L V P Y L Y I N S R T 660

1981
GGTGTGAGCGCTGATGTGGACGGGGGCAATGACCCTCACGATGCCATTTTGCGTATTGCC 2040
661 G V S A D V D G G N D P H D A I L R I A 680

2041 ATTGTAGCATTTGGACCTATTGGTATCAATGCCGGTGTTGCTGCTGTTTTCTTTGGTATG
2100
681 I V A F G P I G I N A G V A A V F F G M 700

F I G. 10E

2101 GCATGCTGCATGGGTCCCATCCTGAGCATGTGCTGCAAGAAGTTCGGTGCTGTGTTGGCG 2160
701 A C C M G P I L S M C C K K F G A V L A 720

2161 GCTATTGCCCACGCGATTGCTGTGATCATCTTGCTTGTCATCTTTGAAGTCATGTTCTTC 2220
721 A I A H A I A V I I L L V I F E V M F F 740

2221 CTCGAACACTGGTCTTGGCCCCGGTGCGTCATGGGCATGATCGCCATGGGTGCCATTCAA 2280
741 L E H W S W P R C V M G M I A M G A I Q 760

2281 CGTTTCGTCTACAAACTTATTATCGCGCTCGCTCTTACCCGAGAGTTCAAGCATGACCAG 2340
761 R F V Y K L I I A L A L T R E F K H D Q 780

2341 TCGAACATCGCATGGTGGACTGGAAAATGGTACAACATGGGTTGGGACTCTCTCTCTCAA 2400
781 S N I A W W T G K W Y N M G W D S L S Q 800

2401 CCGGGCCGAGAGTTCCTCTGCAAGATCACGGAGTTGGGCTATTTCTCAGCAGACTTCGTC 2460
801 P G R E F L C K I T E L G Y F S A D F V 820

2461 ATTGGTCATCTCCTATTGTTCATTATGCTGCCCGCTCTTTGTGTTCCTTACATTGACAAG 2520
821 I G H L L L F I M L P A L C V P Y I D K 840

FIG. 10F

2521 TTTCACTCAGYCATTCTCTTTTGGGTCCSGCCCAAGGTAAGAACC 2565
841 F H S X I L F W V X P K V R T 855

FIG. 10G

```
            10          20          30          40          50          60
             *           *           *           *           *           *
GGT ACC ATC TAC TGG ATG TAC ACT GCT TAC AAC TCC CCA ACC TTG TAT ACT AAA CAT TAT

            70          80          90         100         110         120
             *           *           *           *           *           *
GTC CAA ACC ATA AAT CAA CAA CCA CTT GCT TCG TCA AGA TGG GCT GCT TGT GCC ATT GGT

           130         140         150         160         170         180
             *           *           *           *           *           *
GGT GTT CTT GCT TCA TTT ATT CAA ATT CTT GCC ACA CTT TTC GAA TGG ATT TTC GTG CCT

           190         200         210         220         230         240
             *           *           *           *           *           *
AGA GAA TGG GCC GGT GCT CAA CAT TTG AGT CGT CGT ATG CTA TTT TTG GTG TTA ATT TTC

           250         260         270         280         290         300
             *           *           *           *           *           *
TTA CTC AAT TTG GTT CCA CCA GTT TAT ACA TTC CAA ATT ACC AAA TTG GTG ATT TAT TCG

           310         320         330         340         350         360
             *           *           *           *           *           *
AAA TCG GCA TAT GCT GTG TCG ATT GTT GGA TTT TTC ATT GCT GTG GCC ACT TTA GTA TTC

           370         380         390         400         410         420
             *           *           *           *           *           *
TTT GCC GTC ATG CCA TTG GGT GGT TTA TTC ACT TCA TAC ATG AAC AAG AGA TCA AGA AGA

           430         440         450         460         470         480
             *           *           *           *           *           *
TAT ATT GCA TCA CAA ACA TTT ACT GCC AAC TAC ATT AAA TTG AAA GGT TTA GAT ATG TGG

           490         500         510         520         530         540
             *           *           *           *           *           *
TAT ATT GCA TCA CAA ACA TTT ACT GCC AAC TAC ATT AAA TTG AAA GGT TTA GAT ATG TGG

           550         560         570         580         590         600
             *           *           *           *           *           *
ATG TCT TAT TTG TTA TGG TTT TTG GTT TTC CTT GCC AAA TTG GTT GAA TCT TAT TTC TTC
```

FIG.11A

610 620 630 640 650 660

TTG ACT TTG TCT TTA AGA GAT CCT ATT AGA AAC TTG TCG ACC ATG ACA ATG AGA TGT GTT

670 680 690 700 710 720

GGT GAA GTT TGG TAC AAA GAT ATT GTT TGT AGA AAC CAA GCC AAG ATT GTC TTG GGG TTG

730 740 750 760 770 780

ATG TAT CTT GTT GAT TTG TTA TTG TTC TTT TTG GAT ACT TAT ATG TGG TAC ATT ATT TGT

790 800 810 820 830 840

AAC TGT ATC TTC TCC ATT GGT CGT TCA TTC TAT TTG GGT ATT TCC ATT TTG ACT CCT TGG

850 860 870 880 890 900

AGA AAC ATT TTC ACC AGA TTG CCA AAG AGA ATT TAT TCC AAG ATT TTA GCT ACC ACG GAA

910 920 930 940 950 960

ATG GAA ATC AAA TAT AAA CCT AAA GTT TTG ATT TCA CAA ATT TGG AAT GCC ATT GTT ATT

970 980 990 1000 1010 1020

TCC ATG TAC AGA GAA CAC TTG TTA GCC ATT GAT CAC GTT CAA AAA TTA TTG TAT CAT CAA

1030 1040 1050 1060 1070 1080

GTC CCA TCT GAA ATT GAA GGT AAG AGA ACT TTG AGA GCT CCA ACT TTC TTT GTT TCT CAA

1090 1100 1110 1120 1130 1140

GAT GAC AAC AAT TTT GAA ACG GAA TTT TTC CCA AGA AAT TCT GAA GCT GAA AGA AGA ATT

1150 1160 1170 1180 1190 1200

TCA TTT TTC GCT CAA TCT TTG GCT ACA CCA ATG CCA GAA CCA TTA CCA GTT GAT AAT ATG

FIG.11B

```
         1210         1220         1230         1240         1250         1260
           *            *            *            *            *            *
CCA ACT TTT ACT GTT TTT ACT CCT CAT TAT TCG GAA AAG ATT TTG TTA TCT TTG AGA GAA

         1270         1280         1290         1300         1310         1320
           *            *            *            *            *            *
ATC ATT AGA GAA GAT GAT CAA TTC TCA AGA GTG ACA TTA TTG GAA TAT TTG AAA CAA TTA

         1340         1350         1360         1370         1380         1390
           *            *            *            *            *            *
CAT CCA GTT GAA TGG GAT TGT TTT GTT AAG GAC ACC AAG ATT TTG GCT GAA GAA ACT GCT

         1400         1410         1420         1430         1440         1450
           *            *            *            *            *            *
GCT TAT GAA AAT GGT GAT GAT TCT GAA AAA TTA TCT GAA GAT GGA TTG AAA TCC AAG ATT

         1460         1470         1480         1490         1500         1510
           *            *            *            *            *            *
GAT GAT TTA CCA TTC TAT TGT ATT GGT TTC AAG TCT GCC GCC CCT GAA TAT ACT TTA AGA

         1520         1530         1540         1550         1560         1570
           *            *            *            *            *            *
ACA AGA ATT TGG GCT TCA TTG AGA TCC CAA ACT TTG TAC AGA ACT GTA TCT GGG TTT ATG

         1580         1590         1600         1610         1620         1630
           *            *            *            *            *            *
AAT TAT GCC AGA GCC ATT AAA TTG TTA TAC AGA GTG GAA AAC CCA GAA TTG GTT CAA TAT

         1640         1650         1660         1670         1680         1690
           *            *            *            *            *            *
TTC GGT GGT GAT CCT GAA GGA TTA GAA TTA GCT TTA GAA AGA ATG GCC AGA AGA AAG TTT

         1700         1710         1720         1730         1740         1750
           *            *            *            *            *            *
AGA TTT TTG GTT TCT ATG CAA AGA TTG TCT AAA TTC AAA GAT GAT GAA ATG GAA AAT GCT

         1760         1770         1780         1790         1800         1810
           *            *            *            *            *            *
GAG TTC TTA TTG CGT GCT TAC CCT GAT TTG CAA ATT GCT TAC TTG GAT GAA GAA CCG GCT
```

FIG.11C

```
        1820      1830      1840        1850      1860      1870
          *         *         *           *         *         *
TTG AAT GAG GAC GAG GAA CCA AGA GTA TAC TCT GCC TTG ATT GAT GGT CAT TGT GAA ATG

        1880      1890      1900        1910      1920      1930
          *         *         *           *         *         *
TTA GAA AAT GGT AGA CGT CGT CCT AAA TTC AGA GTT CAA TTG TCT GGT AAT CCA ATT TTG

        1940      1950      1960        1970      1980      1990
          *         *         *           *         *         *
GGT GAT GGT AAA TCT GAT AAT CAA AAT CAT GCG GTT ATT TTC CAT AGA GGT GAA TAT ATT

        2000      2010      2020        2030      2040      2050
          *         *         *           *         *         *
CAA TTG ATT GAT GCT AAT CAA GAT AAT TAT TTG GAA GAA TGT TTG AAG ATT AGA TCA GTT

        2060      2070      2080        2090      2100      2110
          *         *         *           *         *         *
TTG GCT GAA TTT GAA GAA ATG AAT GTT GAA CAT GTT AAT CCA TAT GCA CCA AAT TTG AAA

        2120      2130
          *         *
TCT GAA GAT AAT AAC ACC AAG AAG GAT CC
```

FIG.11D

10 20 30 40 50 60
Gly Thr Ile Tyr Trp Met Tyr Thr Ala Tyr Asn Ser Pro Thr Leu Tyr Thr Lys His Tyr

70 80 90 100 110 120
Val Gln Thr Ile Asn Gln Gln Pro Leu Ala Ser Ser Arg Trp Ala Ala Cys Ala Ile Gly

130 140 150 160 170 180
Gly Val Leu Ala Ser Phe Ile Gln Ile Leu Ala Thr Leu Phe Glu Trp Ile Phe Val Pro

190 200 210 220 230 240
Arg Glu Trp Ala Gly Ala Gln His Leu Ser Arg Arg Met Leu Phe Leu Val Leu Ile Phe

250 260 270 280 290 300
Leu Leu Asn Leu Val Pro Pro Val Tyr Thr Phe Gln Ile Thr Lys Leu Val Ile Tyr Ser

310 320 330 340 350 360
Lys Ser Ala Tyr Ala Val Ser Ile Val Gly Phe Phe Ile Ala Val Ala Thr Leu Val Phe

370 380 390 400 410 420
Phe Ala Val Met Pro Leu Gly Gly Leu Phe Thr Ser Tyr Met Asn Lys Arg Ser Arg Arg

430 440 450 460 470 480
Tyr Ile Ala Ser Gln Thr Phe Thr Ala Asn Tyr Ile Lys Leu Lys Gly Leu Asp Met Trp

490 500 510 520 530 540
Met Ser Tyr Leu Leu Trp Phe Leu Val Phe Leu Ala Lys Leu Val Glu Ser Tyr Phe Phe

550 560 570 580 590 600
Leu Thr Leu Ser Leu Arg Asp Pro Ile Arg Asn Leu Ser Thr Met Thr Met Arg Cys Val

FIG.12A

610 620 630 640 650 660

Gly Glu Val Trp Tyr Lys Asp Ile Val Cys Arg Asn Gln Ala Lys Ile Val Leu Gly Leu

670 680 690 700 710 720

Met Tyr Leu Val Asp Leu Leu Leu Phe Phe Leu Asp Thr Tyr Met Trp Tyr Ile Ile Cys

730 740 750 760 770 780

Asn Cys Ile Phe Ser Ile Gly Arg Ser Phe Tyr Leu Gly Ile Ser Ile Leu Thr Pro Trp

790 800 810 820 830 840

Arg Asn Ile Phe Thr Arg Leu Pro Lys Arg Ile Tyr Ser Lys Ile Leu Ala Thr Thr Glu

850 860 870 880 890 900

Met Glu Ile Lys Tyr Lys Pro Lys Val Leu Ile Ser Gln Ile Trp Asn Ala Ile Val Ile

910 920 930 940 950 960

Ser Met Tyr Arg Glu His Leu Leu Ala Ile Asp His Val Gln Lys Leu Leu Tyr His Gln

970 980 990 1000 1010 1020

Val Pro Ser Glu Ile Glu Gly Lys Arg Thr Leu Arg Ala Pro thr Phe Phe Val Ser Gln

1030 1040 1050 1060 1070 1080

Asp Asp Asn Asn Phe Glu Thr Glu Phe Phe Pro Arg Asn Ser Glu Ala Glu Arg Arg Ile

1090 1100 1110 1120 1130 1140

Ser Phe Phe Ala Gln Ser Leu Ala Thr Pro Met Pro Glu Pro Leu Pro Val Asp Asn Met

1150 1160 1170 1180 1190 1200

Pro Thr Phe Thr Val Phe Thr Pro His Tyr Ser Glu Lys Ile Leu Leu Ser Leu Arg Glu

FIG.12B

			1210			1220			1230			1240			1250			1260	
Ile	Ile	Arg	Glu	Asp	Asp	Gln	Phe	Ser	Arg	Val	Thr	Leu	Leu	Glu	Tyr	Leu	Lys	Gln	Leu
			1270			1280			1290			1300			1310			1320	
His	Pro	Val	Glu	Trp	Asp	Cys	Phe	Val	Lys	Asp	Thr	Lys	Ile	Leu	Ala	Glu	Glu	Thr	Ala
			1330			1340			1350			1360			1370			1380	
Ala	Tyr	Glu	Asn	Gly	Asp	Asp	Ser	Glu	Lys	Leu	Ser	Glu	Asp	Gly	Leu	Lys	Ser	Lys	Ile
			1390			1400			1410			1420			1430			1440	
Asp	Asp	Leu	Pro	Phe	Tyr	Cys	Ile	Gly	Phe	Lys	ser	Ala	Ala	Pro	Glu	Tyr	Thr	Leu	Arg
			1450			1460			1470			1480			1490			1500	
Thr	Arg	Ile	Trp	Ala	Ser	Leu	Arg	Ser	Gln	Thr	Leu	Tyr	Arg	Thr	Val	Ser	Gly	Phe	Met
			1510			1520			1530			1540			1550			1560	
Asn	Tyr	Ala	Arg	Ala	Ile	Lys	Leu	Leu	Tyr	Arg	Val	Glu	Asn	Pro	Glu	Leu	Val	Gln	Tyr
			1570			1580			1590			1600			1610			1620	
Phe	Gly	Gly	Asp	Pro	Glu	Gly	Leu	Glu	Leu	Ala	Leu	Glu	Arg	Met	Ala	Arg	Arg	Lys	Phe
			1630			1640			1650			1660			1670			1680	
Arg	Phe	Leu	Val	Ser	Met	Gln	Arg	Leu	Ser	Lys	Phe	Lys	Asp	Asp	Glu	Met	Glu	Asn	Ala
			1690			1700			1710			1720			1730			1740	
Glu	Phe	Leu	Leu	Arg	Ala	Tyr	Pro	Asp	Leu	Gln	Ile	Ala	Tyr	Leu	Asp	Glu	Glu	Pro	Ala

FIG.12C

```
           1750      1760      1770       1780      1790      1800
              *         *         *          *         *         *
Leu Asn Glu Asp Glu Glu Pro Arg Val Tyr Ser Ala Leu Ile Asp Gly His Cys Glu Met

           1810      1820      1830       1840      1850      1860
              *         *         *          *         *         *
Leu Glu Asn Gly Arg Arg Arg Pro Lys Phe Arg Val Gln Leu Ser Gly Asn Pro Ile Leu

           1870      1880      1890       1900      1910      1920
              *         *         *          *         *         *
Gly Asp Gly Lys Ser Asp Asn Gln Asn His Ala Val Ile Phe His Arg Gly Glu Tyr Ile

           1930      1940      1950       1960      1970      1980
              *         *         *          *         *         *
Gln Leu Ile Asp Ala Asn Gln Asp Asn Tyr Leu Glu Glu Cys Leu Lys Ile arg Ser Val

           1990      2000      2010       2020      2030      2040
              *         *         *          *         *         *
Leu Ala Glu Phe Glu Glu Met Asn Val Glu His Val Asn Pro Tyr Ala Pro Asn Leu Lys

           2050      2060
              *         *
Ser Glu Asp Asn Thr Lys Lys Asp Pro
```

FIG.12D

List of Strains

Strain Name	Relevant Properties	MY No.	ATCC
YFK0688-14B	MATalpha *fks1-1* (506^S)	none	
YFK0931-03B	MATalpha cnb1::LYS2 fks1-1/pDL1 (506^s)	none	
YFK0931-07B	MATa cnb1::LYS2 fks1-1/pDL1 (506^s)	none	
YFK0931-10C	MATa cnb1::LYS2 fks1-1/pDL1 (506^s)	none	
YFK0932-01C	MATalpha cnb1::LYS2 fks1-1/pDL1 (506^s)	none	
YFK0996-11B	MATa fks1-1 pcr1(fks2-1)/pDL1 (506^s 560^R)	none	
YFK0996-23D	MATa pcr1(fks2-1) cnb1::LYS2	none	
YFF2720	MATalpha fks2::TRP1	none	
YFF2721	MATalpha fks2::TRP1	none	
YFK0978 (YM148)	MATa cnb1::LYS2 fks1-1 pcr1(fks2-1)/pDL1 (506^s 560^R)	MY2256	xxx
YFK1088-23B	MATa pcr1(fks2-1) (560^R)	MY2257	xxx
YFK1088-16D	MATalpha pcr1(fks2-1) (560^R)	MY2258	xxx
YFK1087-20B	MATalpha fks1-1 pcr1(fks2-1) (506^s 560^R)	MY2259	xxx
YFK1087-20A	MATa fks1-1 pcr1(fks2-1) (506^s 560^R)	MY2260	xxx

FIG. 13